NEW SYNAPTIC ORGANIZING PROTEINS AND THEIR ROLES IN EXCITATORY AND INHIBITORY SYNAPSE DEVELOPMENT

by

Katherine Laura Pettem

B.Sc. Hon., The University of Victoria, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2012

Abstract

The brain consists of billions of neurons. During development, these neurons must migrate to their proper position and form connections with neighboring neurons to form networks. The specificity and maturation of these connections, or synapses, are critical for proper brain function, including learning, memory and cognition. Many cell adhesion molecules (CAMs) are involved in the formation and maturation of synapses, including the well-characterized neuroligin-neurexin pair. In this study, two new synapse modifying proteins, calsyntenin and MDGA, are characterized using *in vitro* assays and primary hippocampal neuron cultures. Calsyntenin-3 was identified in an un-biased screen to search for new synaptogenic proteins. It is a post-synaptic transmembrane protein that induces the formation of excitatory and inhibitory presynaptic specializations in contacting axons via extracellular cadherin and LNS domains. Overexpression of calsyntenin-3 in neurons increases presynaptic protein clustering. Interestingly, calsyntenin-3 binds to α-neurexins with high affinity, suggesting presynaptic induction is mediated through trans-synaptic signaling with neurexins. MDGAs are a family of synaptic GPI-linked proteins that bind neuroligin-2 with high affinity. MDGA1 blocks the presynaptic induction activity of neuroligin-2, through blocking binding to neurexins, via extracellular immunoglobulin domains. Overexpression of MDGA1 in neurons specifically decreases inhibitory synapses, while knockdown increases inhibitory synapses. Interestingly, like other synaptic proteins including neurexin and neuroligin, MDGAs have recently been linked to autism spectrum disorders and schizophrenia. Thus, the characterization of the synapse-promoting calsyntenin-3 and the synapse-reducing MDGA1 shed new light

on the mechanisms by which synaptogenesis is regulated. Investigating the complex interplay between molecular players during synaptogenesis is critical not only for understanding normal brain development, but also for providing insight into neurodevelopmental disorders.

Preface

The work presented here is largely the product of my own efforts. I wrote the entire manuscript, including both research chapters, with editorial comments from my supervisor, Dr. Ann Marie Craig, and my supervisory committee.

Elements of Chapter 2 are currently being prepared for submission for publication. Michael Linhoff initially discovered calsyntenins in an un-biased expression screen and first cloned calsyntenin-3 in our lab. All subsequent cloning for the new constructs described in Chapter 2 was completed by myself, with the exception of the Myc-C3ΔPCS-CFP, Myc-C3EX-CD8-C3IN and Clstn3-Fc constructs, which were cloned by a Craig lab post doctoral fellow, Dr. Daisaku Yokomaku. All co-culture, neuron overexpression and western blotting experiments were performed and analyzed by myself. The neurexin binding assay was a collaborative experiment between myself and Craig lab research assistant Lin Luo. I prepared all figures and images for presentation. Experiments were designed by myself with guidance from Dr. Ann Marie Craig.

A version of Chapter 3 is currently being prepared for submission for publication. Synaptic activity of MDGA1 was initially discovered by Dr. Daisaku Yokomaku. Dr. Yokomaku cloned all the new constructs presented in Chapter 3, with the exception of constructs under the β-actin promoter, which were cloned by myself in collaboration with Craig lab postdoctoral fellow Dr. Hideto Takahashi. Fusion proteins were prepared in collaboration with Craig lab postdoctoral fellow Dr. Tabrez Siddiqui. All binding, COS expression and neuron expression experiments were performed and analyzed by myself. I prepared all figures and images for presentation. Experiments were designed by myself, with initial input from Dr. Daisaku Yokomaku, and ongoing guidance from Dr. Ann Marie Craig.

The use of rat primary cultured neurons in this research was approved by the UBC Animal Care Committee under protocol number A09-0280.

Table of Contents

List of Figures

List of Abbreviations

Aβ	amyloid β-protein
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
AKAP	A-kinase anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APLP	APP-like protein
APP	amyloid precursor protein
ASD	autism spectrum disorder
BACE	beta-site APP cleaving enzyme or beta-secretase
BDNF	brain-derived neurotrophic factor
CA	*Cornu Ammonis*
CAD	cadherin domain
CAM	cell adhesion molecule
CASK	calcium/calmodulin-dependent serine protein kinase
CASY-1	*C. elegans* calsyntenin ortholog
Cbln	cerebellin
CD47	cluster of differentiation 47
CIRL	calcium-independent receptor of α-latrotoxin
CL1	G-protein coupled receptor CIRL1/Latrophilin-1
Clstn	calsyntenin
CNS	central nervous system
CNV	copy number variant
CpG	cytosine-phosphate-guanine
CTF	C-terminal fragment
DCC	deleted in colorectal carcinoma
EGF	epidermal growth factor
E/I	excitatory / inhibitory ratio
EPSC	excitatory postsynaptic current
ERC	ELKS-Rab6 interacting protein-CAST
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FMRP	Fragile X mental retardation 1 protein
FNIII	fibronectin type III
GABA	gamma-aminobutyric acid
Gabrb3	gamma-aminobutyric acid receptor subunit beta-3
GDNF	glial cell line-derived neurotrophic factor
GEF	guanine nucleotide exchange factor
GFRα1	GDNF family receptor alpha-1
GKAP	guanylate kinase domain-associated protein
GLR-1	*C. elegans* glutamate receptor-1
GluA	AMPA receptor subunit
GluN	NMDA receptor subunit
GPI	glycosylphosphatidylinositol
GRIP	glutamate receptor interacting protein
GTP	guanosine triphosphate
GTPase	enzymes that hydrolyze GTP
ICD	intracellular domain
Ig	immunoglobulin
IL1RAPL1	IL-1 receptor accessory protein-like 1
IPSC	inhibitory postsynaptic current
JIP	JNK-interacting protein

KBS	KLC1-binding segment
KIBRA	kidney and brain expressed protein
KIF	kinesin superfamily proteins
KLC	kinesin light chain
KO	knockout
LacZ	bacterial enzyme β-galactosidase
LAR	leukocyte antigen-related
LNS	laminin/neurexin/sex hormone-binding globulin
LRRTM	leucine-rich repeat transmembrane protein
LTD	long-term depression
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAM	meprin, A5 protein, receptor protein tyrosine phosphatase mu
MDGA	MAM domain containing glycosylphosphatidylinositol anchor
MeCP2	methyl CpG binding protein 2
MEF2	myocyte enhancer factor 2
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptor
Mint	Munc 18 interacting protein; lin-10/X11
mIPSC	miniature inhibitory postsynaptic current
Narp	neuronal activity regulated pentraxin
NCAM	neural cell adhesion molecule
NGL	netrin-G ligand
Nlg	neuroligin
NMDA	*N*-Methyl-D-aspartate
NP	neuronal pentraxin
Npas4	neuronal PAS domain protein 4
NPR	neuronal pentraxin receptor
NTRK	neurotrophic tyrosine receptor kinase
Nxph	neurexophilin
PCR	polymerase chain reaction
PCS	primary cleavage site
PDZ	postsynaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein
PICK	protein interacting with C-kinase-1
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PTPR	protein tyrosine phosphatase receptor
RIM	Rab3-interacting molecule
Robo	Roundabout
SALM	synaptic adhesion-like molecule
SAM68	Src-associated in mitosis 68 kDa protein
SAP	synapse-associated protein
SHANK	SH3 and multiple ankyrin repeat domains protein
shRNA	short hairpin ribonucleic acid
siRNA	small interference ribonucleic acid
SIRPα	signal regulatory protein alpha
SNAP	synaptosomal-associated protein
SNP	single nucleotide polymorphism
SS	splice site
S-SCAM	synaptic scaffolding molecule
SynCAM	synaptic cell adhesion molecule
TM	transmembrane
TPR	tetratricopeptide repeat
Trk	receptor tyrosine kinase
TSP	thrombospondin

Ube3a	E3 ubiquitin ligase
UNC5	uncoordinated locomotion-5
VGAT	vesicular GABA transporter
VGlut1	vesicular glutamate transporter 1

Acknowledgements

I would like to thank the Michael Smith Foundation for Health Research and the National Science and Engineering Council for financial support during the first four years of my degree. I would also like to thank my supervisory committee members, Dr. Shernaz Bamji, Dr. Lynn Raymond and Dr. Weihong Song, for their scientific input and helpful comments.

I would like to thank my supervisor, Dr. Ann Marie Craig, for her continued mentorship and scientific guidance. I would like to especially express my gratitude for her ongoing support in my pursuit of teaching activities during my degree.

I would like to extend my appreciation to all past and present members of the Craig lab. Thanks to Xiling Xhou for tirelessly preparing my neuron cultures each week. Thanks to Dr. Michael Linhoff and Dr. Daisaku Yokomaku for their respective initial discoveries of calsyntenins and MDGAs and for their ongoing scientific collaboration, as well as to Dr. Hideto Takahashi for his recent help with experiments. Thanks to Lin Luo for so enthusiastically taking over the calsyntenin project, and to Dr. Tabrez Siddiqui for his collaborative efforts and for the hours of "science talk." A very special thanks to my lab-mate and friend, (the freshly graduated) Dr. Frederick Dobie: his scientific advice was always appreciated, but it was his listening ear and joking nature that really got me through long days (and years) at the lab.

I could not have completed this degree without a wonderful support system outside the lab as well. Thanks to Rebecca Taylor for her friendship as we navigated through this "grad school" thing together. Thanks to my dear friend Amanda Garrison, for her constant encouragement, for giving me a daily laugh, and for her patience while we are still "long distance" friends. Thanks to my beautiful, smart and funny sisters, Alison, Emily and Laura Walzak, for always being there for me, and for making me want to be the best big sister I can be.

To my parents, Tim and Cecilia Walzak, words cannot express how much your love and support over the (seemingly endless) years of education mean to me. Whether it was quizzing me for my chemistry test or listening to my late-night worries (Dad), or knowing exactly when I needed to take a break with a power walk or a warm chocolate chip cookie (Mom), your constant encouragement and example have made me the woman I am today. Thank you.

Last, I would like to express my deepest gratitude for my husband, Fernando Pettem. Your unwavering love and tremendous patience are what carried me through this degree. There were times when I "fell behind" and you sure "wait(ed) for me." I am so excited to start our next adventure together!

Dedicated to my parents:

For everything I have become, because of everything you both are.

Chapter 1: Introduction

The vertebrate central nervous system (CNS) consists of billions of neurons. During development, these neurons must migrate to their proper position and form connections with the appropriate target neurons to form large networks. The specificity of these connections, or synapses, is critical for proper brain function; even after the initial contact, both the strength and number of synapses are continually modified. This modification is generally referred to as synaptic plasticity, and is important for learning, memory and cognition in the mature brain. Thus, the formation and stabilization of synapses plays a pivotal role in the overall functioning of the CNS. This introduction will review the major events and molecular players involved in the development of vertebrate CNS synapses, with a focus on the cell adhesion molecules (CAMs) neuroligins and neurexins. In addition, synaptic links to neurodevelopmental disorders will be discussed. In this thesis, new synaptic organizing roles for the proteins calsyntenins and MDGAs (MAM domain containing glycosylphosphatidylinositol anchor) are described, thus the introduction will also review previous work on these proteins.

1.1 Structure and Development of CNS Synapses

1.1.1 Ultrastructure of the Synapse

The majority of the connections in the CNS between neurons are chemical synapses, in which the axon of the presynaptic neuron, containing neurotransmitter release machinery, is tightly apposed to the dendrite of the postsynaptic neuron, which contains neurotransmitter-gated ion channels (Peters et al., 1991). Electrical

action potentials travel down the axon and arrive at the axon terminal, where voltage-gated ion channels trigger the release of neurotransmitters into the synaptic cleft (Katz and Miledi, 1965). There, released neurotransmitters bind to dendritic neurotransmitter receptors and induce changes in postsynaptic membrane potential, increasing or decreasing the likelihood of triggering a new action potential (Cowan et al., 2001).

There are two main types of synapses in the mammalian CNS: excitatory and inhibitory. Excitatory synapses release the neurotransmitter glutamate, which binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-Methyl-D-aspartate (NMDA) receptors (AMPARs and NMDARs), resulting in depolarization of the postsynaptic compartment and increasing the likelihood of generating a new action potential (Watkins and Evans, 1981). AMPA receptors are cation channels that are composed of tetramers of various GluA subunits (GluA1-4) and mediate the fast component of neurotransmission (Greger et al., 2007). NMDA receptors are cation channels composed of tetramers of two GluN1 subunits with two GluN2A-D subunits, require both glutamate and glycine, and depolarization of the postsynaptic cell to open, and mediate slower kinetics in neurotransmission (Paoletti and Neyton, 2007). Inhibitory synapses in the brain release the neurotransmitter gamma-aminobutyric acid (GABA) or glycine, which bind to $GABA_A$ receptors ($GABA_AR$) or glycine receptors, respectively, resulting in hyperpolarizing of the postsynaptic compartment and a decreased chance of a new action potential being generated. $GABA_A$ receptors are ligand-gated chloride channels and are composed of five subunits chosen from various isoforms (α, β, γ, δ, ε, π, θ) (Davies et al., 1996).

Although $GABA_A$ receptors are generally inhibitory, they mediate excitatory transmission early in development due to higher intracellular chloride concentrations (Rivera et al., 1999). Glycine receptors have similar structure to $GABA_A$Rs. AMPA, NMDA and GABA receptor subunit composition determines receptor kinetics and conductance properties, as well as trafficking to synapses; thus subunit expression is both spatially and developmentally regulated (Davies et al., 1996; Greger et al., 2007; Paoletti and Neyton, 2007).

Individual synapses have specialized ultrastructural compartments to carry out neurotransmission (Gray, 1963; Kim et al., 2006; Palay, 1956b). The presynaptic side is characterized by boutons, which are small variscosities (~1 micron) studding the length of axons and are filled with clear synaptic vesicles containing neurotransmitters (Birks et al., 1960; Palay, 1956a). When a depolarizing action potential reaches the bouton, calcium entering through voltage-gated ion channels increases the probability that a vesicle docked at the membrane fuses and releases neurotransmitter into the synaptic cleft (Katz and Miledi, 1965), a ~20-25 nm space between the pre- and postsynaptic cells (Schikorski and Stevens, 1997; Whittaker and Gray, 1962). This fusion occurs specifically at the active zone (Couteaux and Pecot-Dechavassine, 1970), where synaptic vesicles are docked at the membrane. The presynaptic bouton also includes synaptic vesicles embedded in an electron-dense matrix of proteins called the presynaptic web (Akert, 1971; Burns and Augustine, 1995; Hirokawa et al., 1989b; Landis, 1988; Phillips et al., 2001). At excitatory synapses, the dendritic postsynaptic membrane is directly apposed to the active zone of the bouton and is characterized by an electron-dense meshwork of

proteins called the postsynaptic density (PSD) (Garner et al., 2002; Palay, 1956b; Sheng, 2001). The PSD ensures that voltage-gated ion channels, neurotransmitter receptors and other second-messenger signaling molecules are clustered at high densities apposed to the neurotransmitter-releasing active zone (Garner et al., 2002; Palay, 1956b; Sheng, 2001). These types of molecules are also clustered at the postsynaptic terminals of inhibitory synapses, although this region is much less dense when visualized by electron microscopy compared to excitatory PSDs (Colonnier, 1968; Peters and Palay, 1996). Excitatory, or glutamatergic, synapses are generally found on the spines of many dendrites, while inhibitory, or GABAergic, synapses are usually found directly on dendritic shafts or on the cell soma (Gray, 1959). Proteins called cell adhesion molecules (CAMs) extend across the synaptic cleft from both the pre- and postsynaptic sides, and are thought to hold the active zone and postsynaptic terminal in tight association (McAllister, 2007). The elucidation of these basic aspects of synaptic structure and function was the result of the concerted efforts of a number of pioneers in the field, and this section is only a brief overview. For a more thorough description of the historical evolution of theories on synaptic development, please see “A Brief History of Synapses and Synaptic Transmission” (Cowan et al., 2001).

1.1.2 Introduction to the Hippocampus

Although the new work presented in this thesis uses dissociated hippocampal neurons as a model system, it is useful to understand the structure and organization of the intact hippocampus when considering expression patterns of key proteins and potential *in vivo* significance of the results. The hippocampus is part of the limbic

system in the brain and is located in the medial temporal lobe beneath the cortical surface. The hippocampus is composed of two sheets of cells folded in on each other: the dentate gyrus and Ammon's horn. Ammon's horn contains three regions of neurons: CA1, CA2, CA3 (Andersen, 2007). Neurotransmission in the hippocampus follows a three-synapse, unidirectional path, in which input from the entorhinal cortex travels along the perforant path to the dentate gyrus and the CA3. Mossy fibers also provide input to CA3 via the dentate gyrus. CA3 neurons send their axons to CA1 neurons via the Schaffer collateral pathway (Andersen, 2007). The perforant path also provides direct input to CA1 neurons, and these synapses are located on distal apical dendrites. Glutamatergic neurons in the hippocampus have a stereotyped pyramid shape and are called pyramidal neurons, while interneurons in this region are inhibitory GABAergic neurons.

1.1.3 General Stages of Synapse Development

The development of synapses, or synaptogenesis, involves a number of protracted steps. Early events include neuronal differentiation and migration, and have been reviewed elsewhere (Marin and Rubenstein, 2003; Ming and Song, 2005). Next, axonal pathfinding ensures that axons find their correct targets, and plays a major role in determining synaptic specificity. This mostly occurs via chemoaffinity mechanisms, by which diffusible factors help guide axons by binding to receptors on these axons (Figure 1.1, A) (Charron and Tessier-Lavigne, 2005; Skutella and Nitsch, 2001; Tessier-Lavigne, 2002). Diffusible factors are also involved in axon and dendrite arbor development - thus priming axons and dendrites so that they are competent to form synapses. Axon and dendrite arbor development

could be considered the last step before synapse development, or as the first step of synapse development, as these processes are often coupled and arbor development can control synaptic partner choice. There is evidence to suggest two mechanisms help control synapse selection at this stage as axon growth cones contact dendrite neuropil: specific recognition molecules may induce the formation of synapses, and/or the axon may form a number of connections and later eliminate the wrong ones (Dityatev and El-Husseini, 2006). Initial contact is certainly stabilized by CAMs that act as “adhesive” factors (Figure 1.1, B). The molecular players governing axon guidance and competence, and axon-dendrite adhesion will be described in more detail in section 1.2.

1.1.3.1 Synaptic Assembly and Maturation

Once an initial contact has been stabilized, a large number of “inductive” factors orchestrate the transport and clustering of presynaptic vesicles and release machinery, and postsynaptic neurotransmitter receptors and scaffolding molecules through bidirectional trans-synaptic signaling (Figure 1.1, C). These proteins and their roles in synaptic development will be the focus of Section 1.2 below. Inductive factors can mediate fairly rapid assembly of synapses, which requires the membrane trafficking of components on both the pre- and postsynaptic sides. On the presynaptic side, this includes transport of small clear-centered vesicles that are synaptic vesicles precursors, as well as larger dense-core vesicles containing scaffold proteins for the active zone, such as piccolo, bassoon and RIM, and proteins required for vesicle exocytosis, such as syntaxin, SNAP25 and N-type voltage-gated calcium channels (Bury and Sabo, 2010; Shapira et al., 2003).

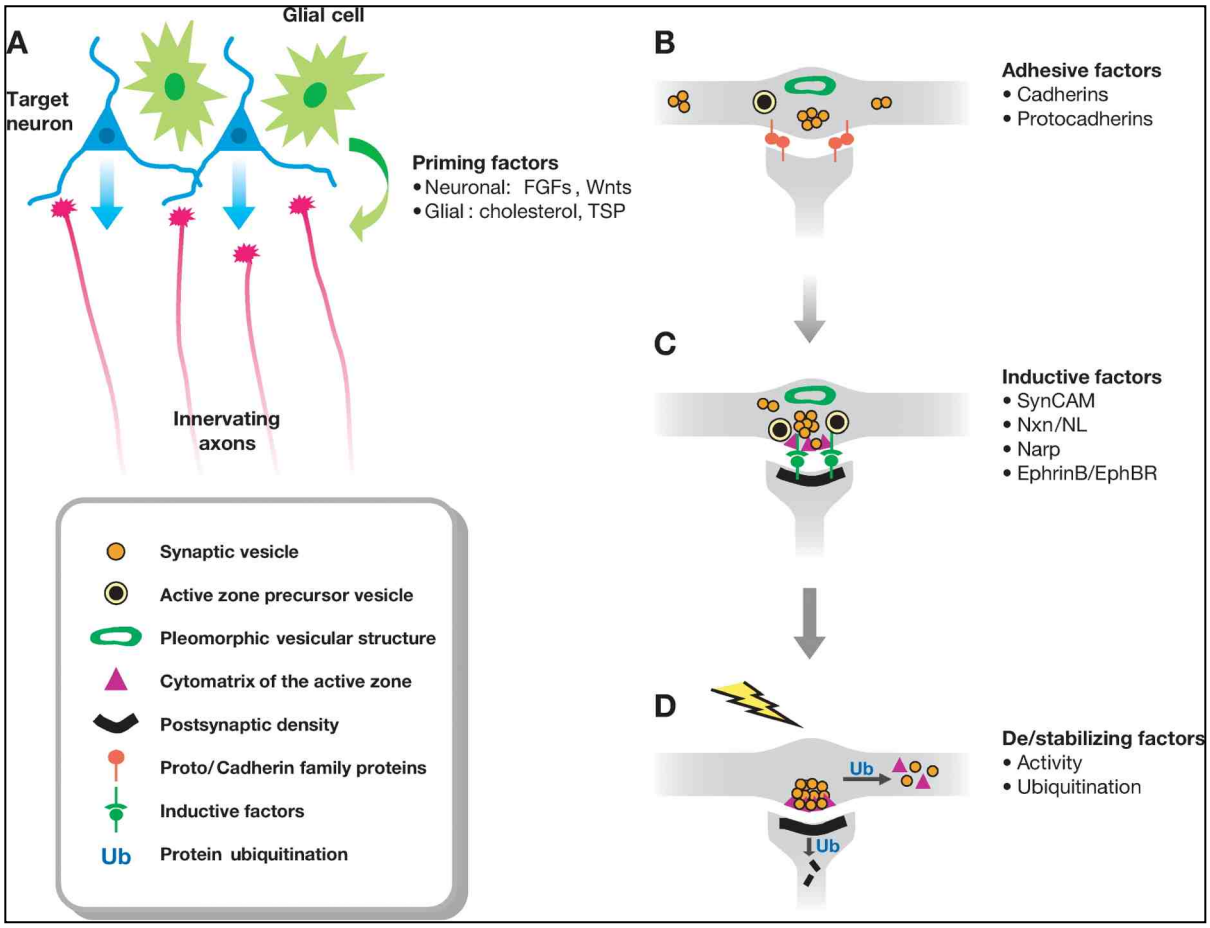

Figure 1.1: Overview of Vertebrate Synaptogenesis
(A) Secreted molecules such as FGFs, Wnts, cholesterol and TSP act as priming factors for innervating axons, promoting axonal and dendritic maturation. (B) Initial contact is facilitated and strengthened by CAMs, including members of the cadherin and protocadherin family. (C) A second group of CAMs act as inductive factors to cluster neurotransmitter release machinery and other active zone proteins on the presynaptic side, and neurotransmitter receptors and postsynaptic scaffolding proteins on the postsynaptic side. Inductive factors include SynCAM, neurexin and neuroligins (Nrx/NL), Narp, EphrinB/EphBR, as well as others (see Figure 1.3). (D) Once synapses are formed, stability is influenced by neuronal activity. Intracellular signaling pathways, such as ubiquitin-mediated degradation, also play roles in the turnover of synaptic components and in synapse elimination. Figure reproduced with permission from Waites et al., Annual Review of Neuroscience, 2005.

Assembly on the postsynaptic side lags slightly behind that on the presynaptic side, and occurs through gradual accumulation of molecules and recruitment of individual components in a sequential fashion. For example, PSD-95 is one of the earliest scaffold proteins recruited to contact sites, and its appearance is followed by the differentially regulated accumulation of both AMPA and NMDA receptors (Waites et

al., 2005). Recruitment can occur through a variety of mechanisms, such as active transport via motor proteins like KIFs, local trapping of diffuse plasma membrane or cystolic pools, or local protein synthesis (McAllister, 2007; Waites et al., 2005). Another point of regulation during synapse formation and assembly is at the level of gene transcription, and a number of transcription factors such as MEF2 and Npas4 have been shown to regulate expression of synaptogenic proteins (Greer and Greenberg, 2008).

In contrast to initial assembly, which can take place in a matter of minutes, maturation of synapses takes place over a more protracted period of time via a series of sequential cues (Ahmari et al., 2000; Craig et al., 2006; McAllister, 2007; Zhai et al., 2001). Synapses expand in a coordinated manner, with pre- and postsynaptic remaining correlated in size (Harris and Stevens, 1989; Pierce and Mendell, 1993; Schikorski and Stevens, 1997). Glutamatergic synapses, which initially form on filopodia or dendritic shafts, undergo a major structural change to develop mature dendritic spine morphology, and take several forms such as mushroom, branches, thin or stubby (McAllister, 2007; Waites et al., 2005). In addition to structural changes, synapses also undergo functional changes as they mature, such as a decrease in the probability of neurotransmitter release and an increase in the reserve pool of vesicles (Bolshakov and Siegelbaum, 1995; Chavis and Westbrook, 2001). Another functional change involves the conversion of “silent synapses;” these synapses are common in developing brain regions and are characterized by functional NMDA currents but not AMPA currents (Durand et al., 1996; Isaac et al., 1997). Conversion occurs via activation of NMDA receptors and

results in recruitment of AMPA receptors to the postsynaptic membrane (Waites et al., 2005).

The overall result of these various structural and functional changes is mature synapses in which presynaptic neurotransmitter-filled vesicles are docked at the active zone, which is apposed to the postsynaptic membrane containing clusters of neurotransmitter receptors; cell adhesion molecules bridge the synaptic cleft to keep the pre- and postsynaptic sides aligned (Figure 1.2). Scaffolding proteins on both pre- and postsynaptic sides help to cluster various components, such as Mint and CASK in axon terminals, PSD-95 in glutamatergic PSDs, and gephyrin in GABAergic PSDs. Glutamatergic synaptic components have been much better characterized than GABAergic components.

1.1.3.2 Stabilizing and Destabilizing Factors

Synapses are dynamic structures, constantly being regulated and modulated in response to neural activity and other environmental signals. This synaptic plasticity can take many forms, but the two that are most well established in the mammalian CNS are long-term potentiation (LTP) and long-term depression (LTD). LTP is characterized by a persistent increase in synaptic strength as a result of patterned input and is thought to be involved in the formation of memory, while LTD is characterized by a persistent reduction in synaptic strength as a result of weak or poorly correlated synaptic input. Although synaptic activity is not required for the basic assembly of synapses (Varoqueaux et al., 2002; Verhage et al., 2000), it does play major roles in determining circuit formation during development (Katz and Shatz, 1996), and later regulates synaptic composition and strength

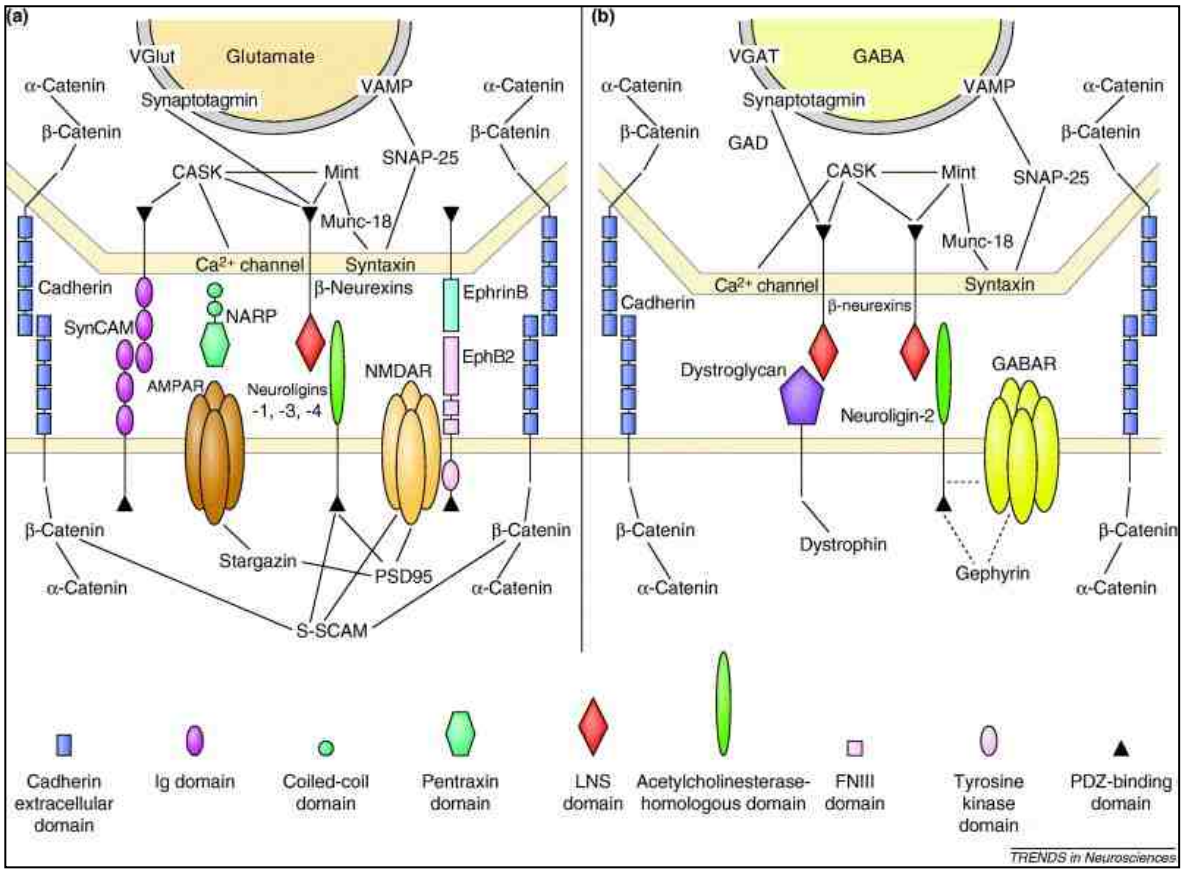

Figure 1.2: Molecular Components of Synapses
Overview of basic components of glutamatergic (a) and GABAergic (b) synapses. Shown is a simplified view, and many other cell adhesion, scaffolding and signaling molecules are also present at synapses. Solid lines represent protein-protein interactions, and broken lines represent presumed indirect interactions. Figure reproduced with permission from Craig et al., Trends in Neurosciences, 2006.

(Bredt and Nicoll, 2003; Craig and Boudin, 2001; Malinow and Malenka, 2002). Synapse elimination also plays a role during maturation (Figure 1.1, D). An excess of synapses are initially formed and activity-dependent pruning of these connections is critical for many aspects of neurodevelopment (Waites et al., 2005). Furthermore, elimination of connections is likely also important in the mature brain in order to fine-tune networks in response to activity. New techniques, such as two-photon transcranial imaging of fluorescently labeled neurons, allow for the real-time monitoring of synapses and are confirming that modification, addition and elimination

of synapses occurs rapidly in response to changes in neural input and activity (Chen and Nedivi, 2010; Fu and Zuo, 2011; Pan and Gan, 2008).

One way that activity may regulate synaptic strength is through up- or down-regulation of synaptic protein expression. Ubiquitination, a chemical modification that targets proteins for degradation, was found to be responsible for the activity-dependent decrease of a few PSD proteins, including SHANK, GKAP and AKAP79/150 (Ehlers, 2003). This modification, however, resulted in the down-regulation of a larger number of PSD proteins, suggesting that the degradation of a few proteins can lead to the rapid destabilization of large synaptic protein complexes and thus synapse stability. Subsequent studies have shown that ubiquitin-mediated protein degradation plays critical roles in regulating synaptic formation, stability and plasticity in both the pre- and postsynaptic compartments (Mabb and Ehlers, 2010; Yi and Ehlers, 2007). Furthermore, mutations and deletions in ubiquitin pathway proteins have also been implicated in neurological and psychiatric diseases, including autism spectrum disorders (ASDs) and Parkinson's (Jiang and Beaudet, 2004; Mabb and Ehlers, 2010).

1.2 Synapse Organizing Secreted and Cell Adhesion Molecules

1.2.1 Axon Guidance and Diffusible Factors

Axon guidance involves diffusible factors secreted from or expressed on target cells or surrounding glia which bind to receptors on axons to attract or repel, such as netrins and their DCC and UNC5 receptors (Kennedy, 2000), semaphorins and their plexin and neuropilin receptors (Pasterkamp and Kolodkin, 2003), slits and

their Robo receptors (Brose and Tessier-Lavigne, 2000), and ephrins and their Eph receptors (Kullander and Klein, 2002), as well as intracellular signaling molecules such as Rho GTPases and downstream effectors (O'Donnell et al., 2009). Interestingly, regulated proteolysis of many of the receptors for these guidance clues is important for induction of downstream signaling and / or signal transduction of the intracellular domain (ICD) to the nucleus for transcriptional activation (O'Donnell et al., 2009). It is important to note that targeting and axon guidance takes place not only at the cellular level, but also at the subcellular level. For example, some interneurons preferentially form inhibitory synapses at distinct subcellular locations, differentiating between dendritic spines, the dendritic shaft, the soma or even the axon initial segment (Somogyi et al., 1998). Various diffusible and adhesive molecular cues are also involved in this type of subcellular targeting.

Other target neuron secreted proteins, such as the Wnt and fibroblast growth factor (FGF) families, may help to spatially restrict synapse formation by inducing regional axon arborization and/or accumulation of recycling synaptic vesicles in innervating axons (Scheiffele, 2003; Waites et al., 2005) (Figure 1.1, A). Recent data have suggested that there is overlap between the functions of secreted proteins, providing evidence that some can also directly induce synaptogenesis rather than play a purely permissive role. For example, some FGFs can directly induce pre- and/or postsynaptic differentiation (Li et al., 2002; Umemori et al., 2004), and knockout mouse studies showed that FGF22 and FGF7 have specific roles in excitatory and inhibitory presynaptic differentiation, respectively (Terauchi et al., 2010). Some Wnt family members also have more direct roles in synaptogenesis

like increasing vesicle clustering and activating downstream signaling cascades, such as Wnt7a and its receptor Frizzled5 (Cerpa et al., 2008; Inestrosa and Arenas, 2010; Sahores et al., 2010; Salinas and Zou, 2008). Wnts have also been implicated in synaptic plasticity (Gogolla et al., 2009). It is important to note that neighboring glia also participate in priming both axons and dendrites to form synapses by secreting factors such as cholesterol (Mauch et al., 2001) and thrombospondins (TSPs) (Ullian et al., 2004).

However, the synaptogenesis induced by these molecules alone is not as complete as that induced by the cell adhesion molecules (discussed below in Section 1.2.3), and may be due to the fact that secreted proteins often act through second messenger/ signaling pathways and regulation of gene transcription, whereas CAMs act primarily through clustering networks of protein-protein interactions (Siddiqui and Craig, 2011). *In vivo*, these various factors likely cooperate to regulate synaptogenesis. In addition to when, where, and how widely secreted factors are released, effects also depend on the spatial and temporal expression of their receptors. In some cases, receptors have only just been identified, such as $\alpha 2\delta$-1 auxiliary calcium channel subunit as the dendritic receptor for TSPs (Eroglu et al., 2009; Xu et al., 2010). Binding of TSPs to dendritic $\alpha 2\delta$-1 promotes the formation of ultrastructurally normal synapses that lack AMPA receptors ("silent synapses"); the mechanism by which this is accomplished has not yet been determined, but may occur by secondary induction of synaptogenic protein gene expression (Christopherson et al., 2005; Eroglu et al., 2009). Further studies on

regulation of receptor expression will help to elucidate the multiple functions of diffusible factors in synaptogenesis.

1.2.2 Axon-Dendrite Adhesion and Cadherins / Protocadherins

Initial contact is stabilized by adhesive factors, such as immunoglobulin (Ig) superfamily members cadherins and protocadherins (Figure 1.1, B). There are ~20 classical cadherins expressed in the CNS, and they are expressed and localized at synapses beginning at the early stages of synaptogenesis (Fannon and Colman, 1996; Shapiro and Colman, 1999; Uchida et al., 1996; Yagi and Takeichi, 2000; Yamagata et al., 1995). Furthermore, different cadherins are expressed in distinctive but overlapping axon populations and their targets, suggesting they play specific roles in matching pre- and postsynaptic partners via homophilic binding. Cadherins clearly play important roles in synapse development and maintenance and have roles at both the pre- and postsynaptic sides. Overexpression of a dominant-negative Neuronal (N)-cadherin results in a decrease in the number of dendritic spines, perturbed clustering of presynaptic proteins and recycling of synaptic vesicles, and a decrease in clustering of PSD-95, a major postsynaptic scaffolding protein (Togashi et al., 2002). The intracellular domain of cadherin binds to catenins (αN-, β- and p120 catenins), which links cadherins to the actin cytoskeleton (Jou et al., 1995). If the interaction between N-cadherin and β-catenin is enhanced, the size of PSD-95 clusters and presynaptic vesicle clusters is increased, as is the frequency of spontaneous excitatory events at synapses (Murase et al., 2002). Presynaptic β-catenin is particularly important for the cadherin-mediated localization of synaptic vesicles (Bamji et al., 2003). Beta-catenin interacts with the PDZ protein Scribble,

and ablation or knockdown of either β-catenin or Scribble results in mislocalization of synaptic vesicles along the axon (Bamji et al., 2003; Sun et al., 2009). Scribble likely mediates synaptic vesicle clustering by acting as a scaffold protein to recruit additional proteins (Sun et al., 2009). The interaction between cadherin and β-catenin is also modulated by dephosphorylation of β-catenin by the tyrosine phosphatase SHP-2 (which itself is activated by phosphorylation by Fer tyrosine kinase), and disrupting this pathway results in the disruption of β-catenin-cadherin interactions and inhibits clustering of synaptic vesicles (Lee et al., 2008a).

On the postsynaptic side, N-cadherin, via catenins, plays important roles in regulating dendritic spine morphology, motility and maturation (Elia et al., 2006; Murase et al., 2002; Togashi et al., 2002). Cadherin binds to β-catenin, which binds to α-catenin, which associates with actin to control actin dynamics (Yamada et al., 2005). Catenins also regulate spine morphology through downstream signaling via Rho GTPases and Rac (Arikkath and Reichardt, 2008; Brigidi and Bamji, 2011). This is an important function, since spine density, morphology and size affects information transfer, plasticity, learning and memory (Bourne and Harris, 2007). In support of a role in plasticity, recent studies have shown that cadherin-mediated adhesion is regulated by activity, and N-cadherin is required for induction of long-term potentiation, a model of learning and memory (Arikkath and Reichardt, 2008). N-cadherin can also regulate AMPA receptor trafficking through its interaction with β-catenin (Nuriya and Huganir, 2006), while β-catenin acts as a scaffold to recruit a number of PDZ proteins, phosphatases and kinases (Arikkath and Reichardt, 2008).

Classical cadherins are negative in typical assays for “inductive” synaptic factors – they are unable to induce clustering of presynaptic components in contacting axons when expressed in non-neuronal cells, and blocking cadherin activity using antibodies or gene mutations results in mistargeted axons and changes in cell architecture but does not block formation of synapses per se (Inoue and Sanes, 1997; Lee et al., 2001; Sara et al., 2005; Takeichi, 1991). However, data is mounting that even blocking the typical “inductive” CAMs does not abolish synapse formation either and has more effects on synapse maturation, suggesting that the classification of adhesive vs. inductive factors (based on cell culture data) is somewhat arbitrary. Thus, in addition to acting as adhesive factors, cadherins and their intracellular partners catenins are also important for synaptic maturation and dendritic morphology regulation though clustering of synaptic proteins and activation of signaling pathways to modulate actin dynamics (Arikkath and Reichardt, 2008).

Protocadherins may also act as adhesive factors early in synaptogenesis. Protocadherins are a large family of genes with region-specific expression in the developing brain, partially localize to synaptic sites, and also undergo alternative splicing (Hirano et al., 2002; Kohmura et al., 1998; Phillips et al., 2003; Wang et al., 2002a; Wang et al., 2002b; Wu and Maniatis, 1999). This variation could potentially encode for the specificity needed in target recognition. It is likely that protocadherins, like classical cadherins, are involved in target recognition rather than induction of synapse formation (Lee et al., 2003). For example, protocadherin-gamma knockout mice show normal early axon outgrowth and synapse formation, but degeneration of specific populations of neurons in the spinal cord at later stages of development

(Wang et al., 2002b). However, further work is needed to fully characterize the roles of the multiple protocadherin isoforms in synaptic development.

Cadherins and protocadherins are not the only Ig superfamily members involved in the early stages of synaptic adhesion and target recognition: neural cell-adhesion molecules (NCAMs), nectins, sidekicks and neurofascin, also mediate synaptic targeting and may play roles in synaptic plasticity (Dalva et al., 2007; Yamagata et al., 2003).

1.2.3 Inductive Synaptic Cell Adhesion Molecules

The CAMs discussed in this section can be classified as “inductive” or “synaptogenic” factors, as they are able to induce presynaptic or postsynaptic differentiation when presented to axons or dendrites, respectively. An initial test for such induction often includes expressing the protein of interest in a non-neuronal cell (such as a fibroblast) and adding these cells to neurons growing in culture; formation of hemi-presynapses (composed of presynaptic proteins and neurotransmitter-filled vesicles in contacting axons) or hemi-postsynapses (composed of postsynaptic components, including neurotransmitter receptors and associated scaffolding and signaling molecules in contacting dendrites) between fibroblasts and contacting neurons is considered a positive result. Synaptogenic CAMs are cleft-spanning complexes with pre- and post-synaptic partners that bind *in trans* across the synapse, and often mediate bidirectional signaling via both protein-protein interactions and activation of signaling cascades, in addition to physically aligning the pre- and postsynaptic sides and contributing to cell adhesion. Evidence is mounting that many of these CAMs have roles that extend beyond development,

including maintenance of dendritic spine morphology, modulation of pre- and postsynaptic protein function and activation of intracellular signaling, ultimately affecting synaptic strength and plasticity (Dalva et al., 2007). Perhaps the best characterized of these complexes is the neurexin-neuroligin pair, which will be discussed extensively in Section 1.3 below. However, there are a large number of other CAMs also involved in synapse induction (Figure 1.3), and some of the main ones will be reviewed here.

1.2.3.1 Narp and NP1

The neuronal activity-regulated pentraxin Narp/NP2 was one of the first glutamatergic synaptogenic factors identified (O'Brien et al., 1999). NP2 and the related NP1 are secreted proteins that can recruit AMPA receptors to contact sites through interaction with the extracellular domain of AMPA receptor subunits via their pentraxin domains (O'Brien et al., 1999; Sia et al., 2007; Xu et al., 2003). In some interneurons, Narp can also cluster NMDA receptors (Mi et al., 2002). It appears that NP1 and NP2 have a specific temporal influence during development, as NP1/2 knockout (KO) mice show deficits in AMPA receptor clustering and AMPA receptor-mediated neurotransmission in early postnatal stages in the developing visual system, but this transmission surpassed wild type levels later in development (Koch and Ullian, 2010; Sia et al., 2007). This data suggests that NP1 and NP2 may play roles in the conversion of silent synapses during development through their influences on AMPA receptors.

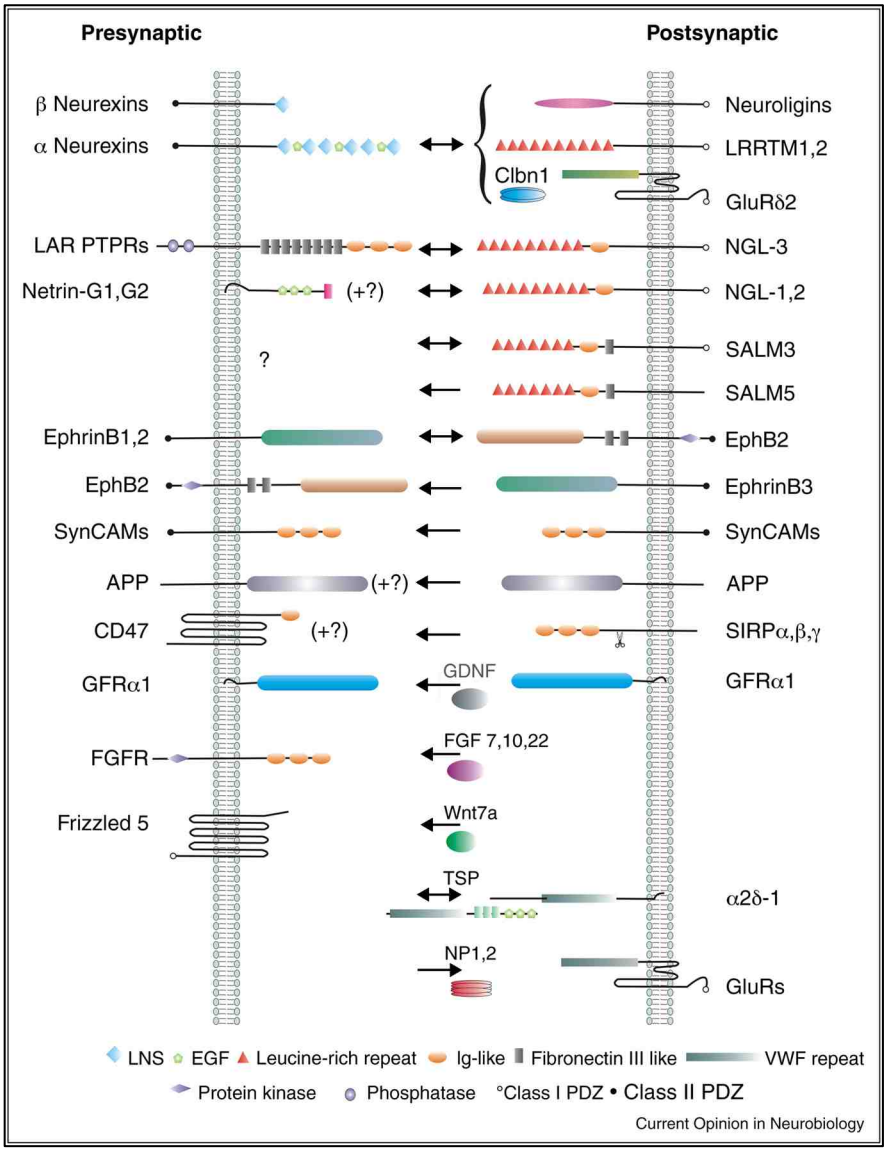

Figure 1.3: Vertebrate Synaptic Organizing Molecules
An inventory of synaptogenic molecules which can induce presynaptic (🡐) or postsynaptic (🡒) differentiation. Bidirectional synaptogenic activity is denoted by (🡐🡒). Main secreted factors and their receptors are also shown. Common protein domains and PDZ binding sites are also displayed. Figure reproduced with permission from Siddiqui and Craig, Current Opinion in Neurobiology, 2011.

1.2.3.2 Ephrins and Eph Receptors

Classically, ephrins and Eph receptors have been associated with axon guidance and boundary formation during development, as both attractive or repellant cues (Kullander and Klein, 2002). New work, however, has shown that EphB2 and

ephrinB3 are also inducers of excitatory hemi-presynapses (Aoto et al., 2007; Kayser et al., 2006; McClelland et al., 2010). EphrinB1/2 and EphB2 are their presynaptic partners, respectively, and both have post synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein (PDZ) domain binding sites in their cytoplasmic domains, allowing them to bind to presynaptic scaffolding proteins such as syntenins and CASK (Siddiqui and Craig, 2011). On the postsynaptic side, dendritic EphB2 can indirectly bind AMPA receptors through PDZ-mediated binding to intermediate proteins like GRIP and PICK1, and can also directly bind the extracellular domain of NMDA receptor subunit GluN1 (Dalva et al., 2000; Kayser et al., 2006). Thus, ephrins and Eph receptors likely act as nucleators for high affinity protein-protein interactions. However, the matter is complicated by the fact that ephrins and Eph receptors are sometimes both expressed pre- and postsynaptically (Lai and Ip, 2009). Furthermore, Eph receptors are receptor tyrosine kinases and bound ephrins themselves are phosphorylated, both of which can activate downstream signaling pathways like tyrosine kinases, GEFs and Rho GTPases; these signaling pathways also play roles in synaptogenesis and spine formation (Kullander and Klein, 2002; Lai and Ip, 2009). It is therefore not surprising that Ephrins and Eph receptors also influence spine morphogenesis and stabilization, and synaptic plasticity (Kayser et al., 2008; Lai and Ip, 2009). This important role in synapse formation and maintenance is supported by the almost complete obliteration of excitatory postsynaptic specializations and dendritic spines in EphB1-3 knockout (KO) mice hippocampal cultures, and defects in PSD size and spine formation *in vivo* (Henkemeyer et al., 2003). Furthermore,

EphB2 single KO mice have a 40% reduction in synaptic NMDAR number and impaired spatial memory, and other Eph and ephrin KO or mutant mice also have various deficits in synapse formation, LTP and tests of learning and memory (Dalva et al., 2007; Henderson et al., 2001).

1.2.3.3 SynCAM1

Synaptic Cell Adhesion Molecule 1 (SynCAM1) is a homophilic transmembrane protein of the immunoglobulin (Ig) superfamily that can induce glutamatergic presynaptic differentiation in culture (Fogel et al., 2007). SynCAM1 has intracellular PDZ-binding domains to link to scaffolding proteins, and directly binds PSD-95 (Biederer et al., 2002). On the presynaptic side, SynCAMs bind CASK/Mint (Biederer et al., 2002). Recent studies suggest that SynCAM1 may also play a role in stabilizing initial adhesion between axonal growth cones and targets, possibly through intracellular interaction with FAK (Stagi et al., 2010). SynCAM1 knockout mice have decreases in excitatory synapse number and transmission, while SynCAM1 overexpressing mice show increases in excitatory synapse number and transmission, suggesting it can directly regulate the formation of synapses (Robbins et al., 2010). At mature synapses, SynCAM1 appears to restrict LTD: SynCAM1 overexpressing mice failed to exhibit LTD, while SynCAM1 KO mice displayed enhanced LTD, which was correlated with enhanced spatial learning (Robbins et al., 2010). Other SynCAM family members, including SynCAM2 and SynCAM3, appear to play roles in axon guidance and myelination (Niederkofler et al., 2010; Park et al., 2008).

1.2.3.4 SALMs

Ig superfamily members SALMs (Synaptic adhesion-like molecules), SALM3 and SALM5, have been shown to induce presynaptic differentiation as well; however, a presynaptic binding partner and mechanism of action has not yet been identified (Mah et al., 2010). However, SALMs can form both heterophilic and homophilic complexes, so induction may occur though binding to SALM4 or SALM5 (Seabold et al., 2008). Overexpression of SALM1 and SALM2 promotes neurite outgrowth and induces dendritic spine formation respectively, but neither can induce presynaptic differentiation in culture (Ko et al., 2006; Wang et al., 2006). Postsynaptic proteins like AMPARs and NMDARs can be recruited by SALM2 and SALM3, and the NMDA receptor subunit GluN1 coimmunoprecipitates with SALM1; these interactions are likely mediated through the PDZ binding domains in SALM1-3 (Ko et al., 2006; Mah et al., 2010; Wang et al., 2006). SALMs may also play roles in glutamatergic synaptic maintenance, as knockdown of SALM2 results in deficits in excitatory, but not inhibitory, neurotransmission in culture (Ko et al., 2006).

1.2.3.5 NGLs, PTPRs and Netrins

Postsynaptic netrin-G ligand (NGL)-3 was first identified as an interacting partner for PSD-95 (Kim et al., 2006). NGL-3 can induce presynaptic differentiation via its leucine-rich repeat domains (Woo et al., 2009). NGL-3 binds the first two fibronectin domains of the presynaptic LAR family of protein tyrosine phosphatase receptors PTPRs: LAR, PTPσ and PTPδ (Kwon et al., 2010; Woo et al., 2009). The intracellular domain of PTPRs is composed of an active phosphatase domain, as well as a domain that binds α-liprins (Pulido et al., 1995). Liprins can bind directly to

scaffolding proteins such as CASK, RIMs and ERC/ELKS/CAST, so it is likely that binding to liprin mediates the ability of PTPRs to induce presynaptic differentiation (Sigrist and Schmitz, 2011; Stryker and Johnson, 2007). It is not known yet whether the phosphatase activity is required for the activities of PTPRs in synaptogenesis. On the postsynaptic side, direct aggregation of NGL-3 on dendrites by antibody-coated beads can mediate excitatory postsynaptic differentiation, by recruiting postsynaptic proteins such as PSD-95, SHANK, GKAP, AMPA receptor subunit GluA2, and NMDA receptor subunit GluN1 (Woo et al., 2009). The mechanism is likely by direct aggregation of PDZ proteins such as PSD-95, PSD-93, SAP102 and SAP97 through the PDZ binding domains of NGL-3 (Kim et al., 2006).

NGL-1 and NGL-2 can also induce excitatory presynaptic specializations, but not as strongly as NGL-3 (Kim et al., 2006). NGL-2 can directly bind to PSD-95, and recruits NMDARs, but not AMPARs in co-culture (Kim et al., 2006). NGL-1 and -2 do not bind LAR, but rather bind netrin-G1 and netrin-G2, respectively; however, since direct aggregation of netrins-Gs on the axon surface does not induce presynaptic differentiation, other yet unidentified co-factors must be required (Kim et al., 2006).

1.2.3.6 TrkC and PTPσ; Slitrk3 and PTPδ

Neurotrophin receptor tyrosine kinase (Trk) C has recently been reported as a new postsynaptic binding partner for PTPσ (Takahashi et al., 2011). Noncatalytic TrkC was identified in an unbiased screen for proteins able to induce hemi-presynapses, and further characterization showed that all TrkC isoforms, but not TrkA or TrkB, bind axonal PTPσ to induce bidirectional signaling in glutamatergic synaptogenesis (Takahashi et al., 2011).

It appears that different PTP family members may play distinct roles in excitatory versus inhibitory synapse formation, as a new study implicated PTPδ in inhibitory synapse development (Takahashi et al., 2012). Axonal PTPδ was shown to be a receptor for dendritic Slit and NTRK-like family member 3 (Slitrk 3), and this trans-synaptic interaction was shown to specifically regulate inhibitory, but not excitatory, synaptogenesis (Takahashi et al., 2012). This finding is particularly interesting considering that the majority of CAMs identified act specifically at glutamatergic synapses, and much less is known about GABAergic synapse development. However, other recent studies implicate PTPδ in excitatory synapse formation via trans-synaptic interaction with both IL-1 receptor accessory protein-like 1 (IL1RAPL1), and interleukin-1 receptor accessory protein (IL-1RAcP) (Valnegri et al., 2011; Yoshida et al., 2012; Yoshida et al., 2011). Further studies may focus on how alternative splicing or other regulatory mechanisms allows for specificity in PTP-mediated synaptogenesis.

1.3 Neurexins and Neuroligins

Originally, neurexins were discovered as receptors for α-latrotoxin, a black widow spider neurotoxin that causes massive neurotransmitter release (Ushkaryov et al., 1992). Intense interest was recently ignited by the study that revealed neuroligins as the first “inductive” synaptic CAM, able to induce presynaptic differentiation in contacting axons when presented on non-neuronal cells (Scheiffele et al., 2000). Shortly thereafter, neurexins, the presynaptic binding partners for neuroligins, were shown to have complimentary activities, inducing postsynaptic

specializations in contacting dendrites when presented on non-neuronal cells (Graf et al., 2004). Since then, this synaptic protein pair has been extensively characterized, and results indicate that neurexin-neuroligin bidirectional signaling plays key roles in the organization of both glutamatergic and GABAergic synapses.

1.3.1 Structure and Expression Patterns of Neurexins and Neuroligins

1.3.1.1 Neurexins

There are three neurexin genes in mammals which are highly conserved across species. Alternative promoter usage results in production of the longer α- and the shorter β-neurexin isoforms (Rowen et al., 2002; Tabuchi and Sudhof, 2002). Alpha-Neurexins contain six Laminin/neurexin/sex hormone-binding globulin (LNS) domains (LNS1-6) interspersed with three epidermal growth factor-like (EGF) repeats, followed by a highly glycosylated region, a transmembrane (TM) domain and a short cytoplasmic tail containing a PDZ binding domain (Figure 1.4). Beta-Neurexins contain a short β-neurexin-specific sequence, followed by the same sequence of α-neurexins beginning at LNS6, and can thus be considered N-terminally truncated α-neurexins. The α-Neurexins contain five extracellular splice sites (denoted SS1-5), with the last two splice sites (SS4 and SS5) shared by β-neurexins (Chubykin, 2009). Splicing inserts from just a few to up to 191 amino acid residues at each site, and splicing at SS5 can even produce secreted forms of neurexin-3 (Missler and Sudhof, 1998). Alternative splicing can theoretically give rise to >2000 neurexin variants (Tabuchi and Sudhof, 2002); the functional significance of some of these splice variants will be discussed in detail in Section 1.3.2 below.

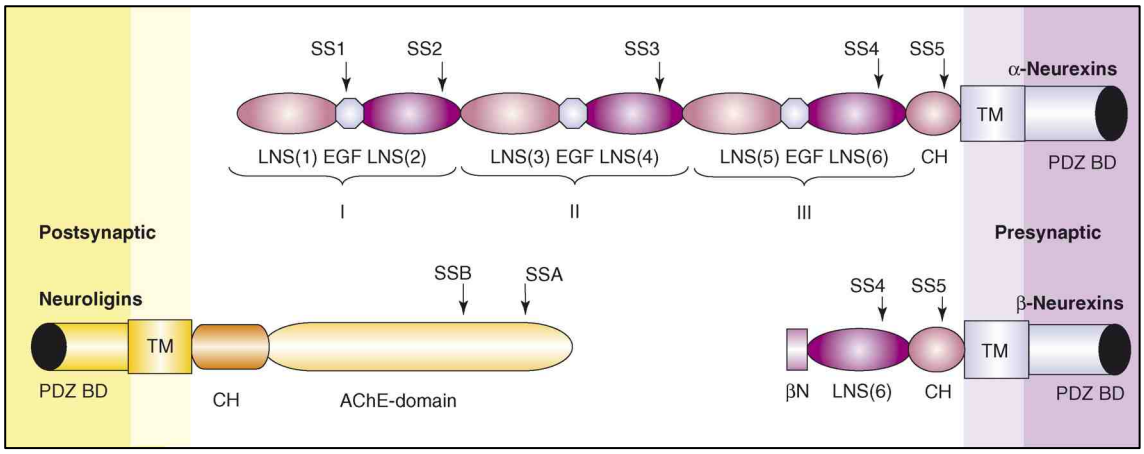

Figure 1.4: Structure of Neurexins and Neuroligins
Presynaptic neurexins are found in the longer α and shorter β form. The α form has six LNS domains separated by three EGF domains, forming three homologous modules (I, II, III), followed by a highly glycosylated region (CH), a single-pass transmembrane domain (TM), and a short intracellular sequence containing PDZ-domain binding domains (PDZ BD). β-Neurexins can be considered N-terminally truncated α-neurexins, and contain a short β-neurexin-specific sequence (βN), followed by and LNS domain corresponding to LNS6 in α-neurexins, the glycosylated region, TM region and intracellular domain. α-Neurexins contain five splice sites in the extracellular domain, labeled as SS1-5; the last two of these sites are also found in β-neurexins. Postsynaptic neuroligins contain an extracellular acetylcholinesterase (AChE)-like domain, a highly glycosylated sequence (CH), followed by a TM domain and a PDZ binding site. Neuroligins have an extracellular splice site (SSA), and a second splice site is found in neuroligin-1 (SSB). Figure modified with permission from Craig and Kang, Current Opinion in Neurobiology, 2007.

The six major neurexins (1α, 1β, 2α, 2β, 3α, 3β) appear to be differentially expressed throughout the brain in overlapping patterns (Ullrich et al., 1995). In summary, neurexin-1α is expressed throughout the brain, with the highest expression in the claustrum, anterior thalamic nuclei and deep cerebellar nuclei, while neurexin-1β expression is mostly confined to cortical layers 2 and 3, the thalamus, and specific parts of the hippocampus. Neurexin-2α is found only in specific subpopulations of layer 2, 4 and 6 cortical neurons, as well as thalamus and cerebellum. Neurexin-2β is more widespread, but is especially high in the superficial layers of the cortex and the cerebellum. Neurexin-3α has quite low expression levels in most brain regions, except for the very superficial and infragranular layers of the

cortex, the striatum, septal nuclei, and the reticular thalamic nucleus. Last, neurexin-3β has uniform expression through most brain regions (Ullrich et al., 1995). In the hippocampus, neurexins also show distinct expression patterns: CA1 pyramidal cells and interneurons lack neurexin-1β, CA1 pyramidal cells and dentate gyrus granule cells show no neurexin-3α expression, while CA3 pyramidal cells express all six major neurexin isoforms. In addition, varying levels of neurexin isoform expression are observed in interneurons in different hippocampal areas, with neurexin-3α showing the highest overall expression (Chubykin, 2009).

Interestingly, specific splice-site isoforms also appear to have spatially regulated expression patterns. In particular, isoforms containing SS4, which regulates the binding specificity to neuroligins (see Section 1.3.2), are higher in striatum, substantia nigra and cerebellar nuclei while isoforms lacking SS4 are higher in CA3 in the hippocampus. In pyramidal cells of CA1-4, the overall most common isoform is neurexin-3(-SS4) (Ichtchenko et al., 1995).

1.3.1.2 Neuroligins

Rodents have four neuroligin genes, while humans have five (Bolliger et al., 2001; Ichtchenko et al., 1996). Interestingly, three of the neuroligin genes are sex-linked, with genes for neuroligin-3 and -4 located on the X-chromosome and neuroligin-5 (sometimes called neuroligin-4Y) on the Y-chromosome in humans (Jamain et al., 2003). Sequence comparison shows that neuroligin-1,-3 and -4/5 are more similar to each other than neuroligin-2. Structurally, neuroligins contain a large N-terminal sequence with homology to the α/β-hydrolase fold of acetylcholinesterase (AChE), a highly glycosylated region, a single-pass transmembrane domain, and a

short intracellular domain containing PDZ binding sites (Ichtchenko et al., 1995) (Figure 1.4). The AChE domain in neuroligins is missing the amino acids needed for catalytic function, as well as the substrate entrance site (Arac et al., 2007; Koehnke et al., 2008); however, this domain does facilitate constitutive dimerization of neuroligin molecules, as it does in acetylcholinesterase (Comoletti et al., 2003). Dimerization is functionally significant, as mutation of the putative dimerization sequences at the binding interface of neuroligin-1 monomers results in elimination of synaptogenic activity (Dean et al., 2003). However, the possible significance of hetero- versus homo-dimerization has not yet been characterized.

Neuroligins have two alternative splice sites located in the AChE domain, SSA and SSB. Neuroligin-1 and -3 have two alternative inserts for the “A” splice site (A1 and A2), whereas all other neuroligins only use A2; splicing at site B has only been observed for neuroligin-1 (Craig and Kang, 2007). All neuroligins are expressed throughout the CNS, with neuroligin-1 specifically localizing to glutamatergic synapses, neuroligin-2 localizing to GABAergic synapses, and neuroligin-3 localizing mostly to glutamatergic but possibly also at some GABAergic synapses (Budreck and Scheiffele, 2007; Levinson et al., 2005; Song et al., 1999; Varoqueaux et al., 2004). Neuroligin-4 is expressed at much lower levels, accounting for less than 5% of the total neuroligins in the mouse brain (Varoqueaux et al., 2006).

1.3.2 Splice Code for Neurexin - Neuroligin Binding

Neuroligins form constitutive dimers, this dimer binds the LNS6 domains (or the single LNS in β-neurexins) of two neurexin molecules, via the AChE domain in

neuroligin (Dean et al., 2003). Binding between neuroligins and neurexins is calcium-dependent and hydrophilic, and thus depends on the ionic concentrations of the environment (Chen et al., 2008). Interestingly, alternative splicing regulates binding specificity in both primary transcripts (Comoletti et al., 2003; Ichtchenko et al., 1995; Ichtchenko et al., 1996), and a number of recent studies have focused on characterizing this relationship between splicing and binding, using both functional studies and crystallization of neurexin-neuroligin complexes. In neurexin, SS2, SS3 and SS4 are all found in LNS domains, and their location places them in loops that surround the Ca^{2+}-binding site which is termed the "hypervariable surface" of LNS domains (Arac et al., 2007; Chen et al., 2008; Fabrichny et al., 2007; Rudenko et al., 2001; Shen et al., 2008). The binding of Ca^{2+} in the center of the hypervariable surface is critical to stabilize the neurexin-neuroligin complex, and mutations in this sequence disrupt the binding between neuroligin and neurexin and therefore eliminate synaptogenic activity (Graf et al., 2006). In neurexins, the binding to neuroligins and the synaptogenic activity is mediated by the same face on LNS6 (the single LNS in β-neurexins), which is also where SS4 is located (Graf et al., 2006). Structural analysis has shown that when the 30 amino acid insert at SS4 is present, the conformation of the extended loop surrounding the Ca^{2+}-binding site changes to provide additional contact points for Ca^{2+} and thus increases the binding affinity to Ca^{2+}. As SS4 is also close to one of the salt bridges formed between neurexin-neuroligin, insertion here likely also changes the topology of the hypervariable surface (Chen et al., 2008). In neuroligin, insertion of SSB has the opposite effect to

SS4 insertion in neurexin, disrupting the salt bridge and obstructing the other rearrangements (Shen et al., 2008).

Surface plasmon resonance studies have shed further light on this issue, showing that for neurexin-1β(-SS4), the strength of binding for neuroligin (Nlg) is Nlg1>Nlg4>>Nlg3>Nlg2 (Comoletti et al., 2006). For binding between neurexin-1β(-SS4) and neuroligin-1, binding is stronger for neuroligin-1(-SSB) than (+SSB), while presence of SSA does not appear to affect binding affinity between neurexin1β(-SS4) and any neuroligins (Comoletti et al., 2006). For neurexin-1β(+SS4), binding is favored for any neuroligins without the SSB (note insert at B site has only been observed for Nlg-1), and is highest for neuroligin-2 (Chih et al., 2006; Graf et al., 2006).

In neuroligin-1, splicing at site B, which involves insertion of eight amino acids, also controls the affinity of binding to neurexins. Neuroligin-1(-SSB) is more permissive and binds both α-neurexins(±SS4) and β-neurexins(±SS4), while neuroligin-1(+SSB) shows selective binding to β-neurexins, with affinity being higher for -SS4 than +SS4 variants, and does not bind α-neurexins(±SS4) (Boucard et al., 2005; Chih et al., 2006). Studies suggest that it is not the insert itself, but rather *N*-linked glycosylation of the insert that changes the binding affinity with neurexin (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2003). With these binding affinities in mind, it is clear that a “splice code” exists to help explain the selective effects for neurexin-neuroligin interactions at excitatory versus inhibitory synapses. Neuroligin-1(+SSB) is the main neuroligin-1 in the adult rat hippocampus, cortex and cerebellum (Chih et al., 2006). Neuroligin-1 and -3 are expressed mostly at

excitatory synapses, where they preferentially bind to neurexin-1β(-SS4), while neuroligin-2 is localized to inhibitory synapses and binds preferentially to neurexin1β(+SS4) and α-neurexins (Budreck and Scheiffele, 2007; Song et al., 1999). However, it is unclear at this time how much splicing determines localization of neuroligins. It has been reported that neuroligin-1(+SSB) localizes preferentially to glutamatergic synapses while neuroligin-1(-SSB) was present equally at glutamatergic and GABAergic synapses (Chih et al., 2006), but artificial addition of SSB to neuroligin-2 did not alter its localization to GABAergic synapses (Graf et al., 2004).

The expression patterns of neurexins correlate well with the splice code, as (+SS4) neurexins are enriched in brain areas with predominantly inhibitory neurons, such as the striatum, substantia nigra and cerebellar nuclei (Oertel and Mugnaini, 1984), while (-SS4) neurexins are found at higher levels in areas with large numbers of excitatory neurons, such as hippocampal pyramidal cells (Ichtchenko et al., 1995). It is important to note that these interactions are not absolute, for example neuroligin-1and -2 can both induce excitatory and inhibitory presynaptic differentiation in culture systems (see Section 1.3.3.1). *In vivo*, many of these functions are likely restricted through regulation of temporal and spatial expression of splice variants, as well as synaptic localization.

The most recent crystallization and surface plasmon resonance studies are providing further support for the specificity observed between different neuroligin and neurexin splice forms. For example, crystallization of a neurexin-1β/neuroligin-4 complex showed that neuroligin-4 requires conformational rearrangements to bind to

neurexin-1β, while neuroligin-1 does not, which could explain the lower binding affinity and higher sensitivity to ionic changes in Nrxn1β/Nlg-4 binding (Leone et al., 2010). Other studies have focused on the structure of α-neurexins, and show how a flexible hinge between LNS5 and EGF3 domains is required to position α-neurexins in an "open" L-shaped form, exposing LNS6 for complex formation with neuroligins (Chen et al., 2011; Miller et al., 2011). Furthermore, insertion of splice site 4 was predicted to change the conformation of neurexin-1α to a "closed" form, which would inhibit binding to neuroligin-1(+SSB), in agreement with other biochemical observations (Boucard et al., 2005; Koehnke et al., 2010; Miller et al., 2011). In addition, steric hindrance was predicted between LNS4(+SS3) of neurexin-1α and SSA of neuroligin-1, suggesting that SSA may also regulate neurexin-neuroligin binding (Chen et al., 2011; Miller et al., 2011). A role for SSA would be supported by earlier studies suggesting that SSA may regulate neuroligin-1-mediated clustering of VGlut1 but not VGAT, as well as localization of neuroligins to GABAergic synapses (Chih et al., 2006). Further functional characterization is needed to fully understand how these multiple splice forms instruct synapse formation, specificity and maturation.

Interestingly, neurexin splicing is modulated by neuronal activity, and a recent study found that the RNA-binding protein SAM68 regulates neurexin splicing changes in response to activity (Iijima et al., 2011). Given the huge possible diversity in neurexin splice variants, it is likely that a number of other factors also regulate the spatial and temporal splice patterns of neurexins.

1.3.3 Evidence for Neurexin and Neuroligins in Synapse Development

1.3.3.1 Co-Culture and Neuron Expression Assays

The first evidence that neuroligins and their binding partners neurexins played roles in synapse development came from the finding that neuroligin-1 could induce presynaptic differentiation in contacting axons from co-cultured pontine neurons when expressed on the surface of non-neuronal cells (Scheiffele et al., 2000). These induced presynaptic terminals were morphologically indistinguishable from those in *bona fide* synapses and were competent for neurotransmitter release. Clustering of neurexins and presynaptic proteins can also be induced by recombinant neuroligins immobilized on beads and presented to axons (Dean et al., 2003). When it was found that direct clustering of epitope-tagged neurexins by antibodies in neurons also induces the clustering of presynaptic proteins, it was hypothesized that perhaps neurexins played a direct role in presynaptic differentiation, and that neurexin-neuroligin signaling could be bidirectional (Dean et al., 2003). This in fact was found to be true, as neurexin expressed on non-neuronal cells or attached to beads induces clustering of neurotransmitter receptors and other postsynaptic components in contacting dendrites (Graf et al., 2004). Furthermore, direct clustering of neuroligins by antibodies also induced postsynaptic differentiation (Graf et al., 2004). These seminal studies positioned the neurexin-neuroligin pair as key mediators of synaptogenesis.

Culture assays also helped establish the splice code and the specificity for neurexins and neuroligins at excitatory and inhibitory synapses. For example, although neuroligin-2 can induce both excitatory and inhibitory presynaptic

differentiation, it is more active on GABAergic axons (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005). In addition, co-cultures with non-neuronal cells expressing neurexin-1β(-SS4) can induce both excitatory and inhibitory postsynaptic markers, but neurexin-1β(+SS4) expressing cells selectively cluster inhibitory markers and neuroligin-2 (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006; Graf et al., 2006). Similarly, co-cultures with neurexin-1α(-SS4) suggest it plays a specific role in GABAergic synaptogenesis, suggesting potential functional overlap with neurexin-1β(+SS4) (Chih et al., 2006; Kang et al., 2008). However, the co-culture assays lack some specificity due to the relatively high level of protein expression on non-neuronal cell surfaces; this may allow even weak interacting partners to induce hemi-synapses. Also, there may be limiting expression of synaptic partners in neuron cultures compared to the situation *in vivo*.

Another way to examine synaptogenic activity is to directly express proteins in cultured neurons. Overexpression of neuroligin-1 in primary dissociated neurons increases the density of both glutamatergic and GABAergic presynaptic terminals, as well as the frequency of miniature excitatory postsynaptic currents (mEPSCs) and excitatory evoked responses (Chih et al., 2005; Levinson et al., 2005). Interestingly, NMDA receptor-mediated currents increased more so than AMPA receptor-mediated currents (Chubykin et al., 2007; Prange et al., 2004). Overexpression of neuroligin-3 also increases both excitatory and inhibitory synaptic terminals onto transfected neurons (Chih et al., 2005). Although neuroligin-2 overexpression increases both excitatory and inhibitory synapse numbers, GABAergic synapses are promoted more strongly (Chih et al., 2005). In addition, overexpression of neuroligin-2 increases the

frequency of miniature inhibitory postsynaptic currents (mIPSCs) and inhibitory evoked responses (Chih et al., 2005; Levinson et al., 2005). However, it is important to note that expression level can have drastic impacts on overexpression study results. For example, very high levels of neuroligin overexpression can actually disperse PSD-95 clusters and results in decreased number of synapses (Graf et al., 2004).

Overexpression has also been a useful tool to examine the neurexin-neuroligin "splice code." For example, neuron overexpression of neuroligin-1(+SSB), or neuroligin-2 with an artificial SSB insert, results in reduced clustering of VGAT, but not VGlut1, compared with neuroligin-1(-SSB) overexpression (Chih et al., 2006). This is consistent with the preferential differentiation of GABAergic synapses by neuroligin-2, which always lacks SSB (Chih et al., 2005; Levinson et al., 2005).

Complimentary to the overexpression approach is to knockdown proteins in culture using short hairpin (sh) or small interfering (si) RNA sequences to decrease expression levels. Knockdown of all three neuroligins in cultured neurons results in a large decrease in amplitude and frequency of mIPSCs, and a smaller decrease in mEPSCs, as well as a drastic decrease in both glutamatergic and GABAergic synapse number (Chih et al., 2005). This finding is supported by the reduced excitatory and inhibitory synapse number and reduced mEPSC and mIPSC frequency observed when soluble β-neurexins are added to neuronal cultures to block endogeneous β-neurexin-neuroligin signaling (Levinson et al., 2005). Knock-down of single neuroligins produce similar, but less severe, phenotypes (Chih et al., 2005).

1.3.3.2 Intracellular Binding Partners

The bidirectional signaling of neurexins and neuroligins to induce synapse formation is mediated by binding to a number of intracellular partners, which likely act by aggregation of proteins in synaptic compartments and/or activation of catalytic signaling. On the presynaptic side, neurexins bind CASK, syntenin and Mints via their class II PDZ binding domain (Biederer and Sudhof, 2000; Grootjans et al., 2000; Hata et al., 1996). CASK, a member of the membrane-associated guanylate kinase (MAGUK) family of adaptor proteins, also binds Veli and Mint scaffolding proteins in a stoichiometric ratio (Butz et al., 1998). Veli also binds β-catenin, thus linking neurexin and cadherin signaling (Perego et al., 2000). CASK, though binding with protein 4.1 and neurexin simultaneously, also nucleates the assembly of actin (Biederer and Sudhof, 2001). CASK also binds Ca^{2+} and K^{+} channels, which may help to cluster them at the presynaptic terminal (Leonoudakis et al., 2004; Maximov et al., 1999). CASK is also a kinase, and phosphorylates the C-terminal region of neurexin in an activity-dependent manner, although the significance of this phosphorylation has not yet been determined (Mukherjee et al., 2008). The phenotype of α-neurexin KO mice (see below) suggests that the interaction between neurexin and CASK is critical for presynaptic Ca^{2+} channel function. Neurexins also may influence synapse development through PDZ-independent interactions, such as binding to the Ca^{2+} sensor synaptotagmin to promote recruitment of synaptic vesicles (Hata et al., 1993).

Retrograde signaling of Neuroligin-1 and PSD-95 can modulate vesicle release probability, and may also play roles in coordinating pre- and postsynaptic

morphological changes (Ehrlich et al., 2007; Futai et al., 2007). On the postsynaptic side, the class I PDZ binding domain in neuroligins facilitates binding to scaffolding proteins such as PSD-95 and synaptic scaffolding molecule (S-SCAM). The PDZ domains of PSD-95 and S-SCAM also bind K^+ channels and NMDA receptors, which may aid in clustering these receptors in the postsynaptic density (Hirao et al., 1998; Irie et al., 1997). PSD-95 binds to stargazin, which links to AMPARs; this interaction is required for AMPA receptor delivery to the postsynaptic membrane (Chen et al., 2000). Neuroligin binding to S-SCAM also links it to cadherin-catenin pathways, as S-SCAM binds to and is localized to synapses by β-catenin (Ide et al., 1999; Nishimura et al., 2002). Neuroligins also cluster NMDARs though a PSD-95-independent mechanism (Chih et al., 2005), although clustering of AMPARs by neuroligins requires additional factors such as activity or perhaps increased levels of PSD-95 (Nam and Chen, 2005). Neuroligins can also bind to SHANKs, which are scaffolding proteins involved in the formation of dendritic spines and recruitment of metabotropic glutamate receptors (Meyer et al., 2004; Sheng and Kim, 2000). A new study suggests that the PDZ-mediated binding is not necessary at all for the functional affects of neuroligin-1 on excitatory neurotransmission, but is instead facilitated via a novel intracellular domain (Shipman et al., 2011), suggesting that other intracellular binding partners still remain to be found. Other known interactions are also PDZ-independent, such as the ability of all neuroligins to bind the GABAergic scaffolding protein gephyrin (Poulopoulos et al., 2009). Only neuroligin-2 binds collybistin, which results in recruitment of gephyrin bound to collybistin, supporting an inhibitory-specific role for neuroligin-2 (Poulopoulos et al., 2009).

Further investigation is required to determine how neuroligin-2 is specifically localized to inhibitory synapses despite its ability to bind glutamate synaptic scaffolding proteins (Craig and Kang, 2007).

1.3.4 Neurexin and Neuroligin Knockout Mouse Models

Studies with knockout mice suggest that, *in vivo*, neuroligins and neurexins have important functions in maturation or activity-dependent stabilization of synapses, but may not play crucial roles in initial synapse formation. The knockout of all three α-neurexins 1-3 (leaving the three β-neurexins intact) results in severe impairments in synaptic transmission, including decreases in evoked inhibitory transmission in the neocortex, evoked excitatory transmission in the brainstem, and drastic reductions in mEPSC and mIPSC frequencies; this knockout is also perinatally lethal (Missler et al., 2003). Excitatory synaptic response deficits were due to NMDAR-mediated, but not AMPAR-mediated, transmission (Kattenstroth et al., 2004). These mice also have a twofold decrease in inhibitory synapses in the brainstem and neocortex at P0, but no change in excitatory synapse number (Missler et al., 2003). This may be explained by the fact that α-neurexins only bind neuroligins(-SSB), so α-neurexin KO may specifically affect the subpopulation of inhibitory synapses bearing neuroligin-2 (always -SSB) which interact with α-neurexins, while the subset of excitatory synapses which express β-neurexins interacting with neuroligin-1(+SSB) would be preserved. Alpha-neurexin KO mice also have severely suppressed presynaptic Ca^{2+} channel currents, although the total number of surface Ca^{2+} channels is not changed (Missler et al., 2003); these effects are possibly mediated though the loss of presynaptic Ca^{2+} channel clustering via α-

neurexin's interactions with CASK. This change in Ca^{2+} channel currents is likely responsible for the dysfunction in synaptic vesicle exocytosis, and resultant postnatal mortality, observed in α-neurexin 1-3 KO mice. Further studies examined double α-neurexin KO mice (1/2 KO or 2/3 KO), which survive longer than the triple KO. Double KO α-neurexin mice that survive to adulthood display a 30% reduction in GABAergic, but not glutamatergic, synapses, as well as a decrease in spine density and dendrite branch length (Dudanova et al., 2007). KO mice for β-neurexins have not yet been reported, but they would likely differ at least somewhat in their phenotype as the deficits in calcium channel function in α-neurexin 1-3 KO mice could not be rescued with transgenic expression of neurexin-1β, showing that α-neurexins are unique in their binding and modulation of calcium channels (Zhang et al., 2005).

Studies with neuroligin single KO and overexpressing mice suggest that neuroligins are involved in maintaining the balance between excitation and inhibition (E/I) in the brain. Neuroligin-1 KO mice show decreased NMDAR-mediated transmission and no change in inhibitory transmission, while neuroligin-2 KO mice show decreased IPSC amplitude and no change in excitatory transmission (Chubykin et al., 2007). The specific NMDAR, but not AMPAR transmission deficits in neuroligin-1 KO mice is also supported by the selective induction of NMDAR, but not AMPAR clustering, by β-neurexin induced clustering of neuroligins, suggesting that neuroligin-1 may be involved in the formation of "silent synapses" (Graf et al., 2004; Nam and Chen, 2005). Acute suppression of neuroligin-1 using short hairpin (sh) RNA in the amygdala also resulted in decreased NMDAR-mediated EPSCs,

decreased NMDA/AMPA ratio, abolished NMDAR-mediated LTP, and deficits in memory tasks (Kim et al., 2008). Deficits in spatial memory are also observed in neuroligin-1 KO mice (Blundell et al., 2010). Further studies showed that neuroligin-2 may have even more selective functions, as KO mice showed specific deficits in perisomatic inhibitory synapses in the hippocampus and at fast-spiking interneuron - pyramidal cell synapses in the cortex (Gibson et al., 2009; Poulopoulos et al., 2009). Neuroligin-2 knockout also increases excitability of granule cells in the cortex, which is likely due to impaired $GABA_AR$ clustering (Jedlicka et al., 2011).

Neuroligin overexpressing mice have also been studied to determine *in vivo* function. Mice that overexpress neuroligin-1 display an in increased maturation of excitatory synapses, a shift in synaptic activity towards excitation, impairments in long-term potentiation (LTP) induction, and deficits in memory acquisition (Dahlhaus et al., 2010). On the other hand, mice that overexpress neuroligin-2 have an increased mIPSC frequency and a decreased E/I ratio, as well as impaired social interactions, anxiety, stereotyped behaviors and increased seizure activity (Hines et al., 2008). Some of these effects on E/I balance may be mediated by neuroligins' interactions with intracellular binding partners. In support of this, overexpression of PSD-95 enhances excitatory transmission, but also reduces inhibitory transmission via translocation of neuroligin-2 from inhibitory to excitatory synapses, without changing the total number of synapses (Levinson and El-Husseini, 2005; Prange et al., 2004).

Triple neuroligin KO mice (negative for neuroligin-1,-2,-3) show much more pronounced deficits than single KOs, and die shortly after birth due to respiratory

failure (Varoqueaux et al., 2006). GABAergic transmission is severely impaired in these mice, but there is no significant decrease in synapse number, suggesting that neuroligins are required for synapse maturation, but not synapse formation *per se* (Varoqueaux et al., 2006). There is also a shift in the E/I balance in the respiratory brainstem towards excitation, as glutamatergic transmission was reduced to a lesser extent than GABAergic transmission. Furthermore, triple neuroligin KO mice have an increased ratio of glutamatergic versus GABAergic synapses in the respiratory brainstem despite having no change in the overall number of synapses (Varoqueaux et al., 2006). The deficits in GABAergic transmission are likely due, at least in part, to altered postsynaptic $GABA_A$ receptor recruitment (Varoqueaux et al., 2006). No changes in gephyrin or PSD-95 were observed, but several changes were detected in synaptic vesicle proteins as well as VGAT/VGlut cluster ratio, implicating presynaptic defects. In contrast to the few known GABAergic synaptic CAMs, there are a large number of other glutamatergic synaptic proteins that may be able to compensate for the complete loss of neuroligins; this may account for the less severe changes in glutamatergic transmission.

Results from KO mice, in which synapse function, but not density, is impaired, may seem inconsistent with culture studies, in which neurexins and neuroligins act as inductive factors in synapse development and knockdown results in decreased numbers of synapses. In co-culture assays, it is possible that a number of synaptic proteins are able to induce clustering of pre- or postsynaptic components when presented at a high concentration by activating downstream signaling pathways; this may not truly reflect their roles *in vivo.* In support of this idea, inhibiting synaptic

activity reduces the ability of neuroligins to increase synapse number when overexpressed in culture, but does not alter expression or localization of neuroligins (Chubykin et al., 2007). In knockdown experiments, single cells are deficient in neuroligin or neurexin in an otherwise wild type culture, whereas KO mice are uniformly lacking neuroligin or neurexin. In the case of single cell knockdown, it is possible that activity-dependent effects - in which functionally disadvantaged neurons lose synapses more easily - result in the observed decrease in synapse number. There are a number of other factors that come into play in the *in vivo* situation, such as possible compensation or redundancy by other synaptogenic factors, and the difficulties in studying synapse formation in animals that die before such processes are complete (Varoqueaux et al., 2006). In light of KO mouse phenotypes, and considering the relatively low binding affinity of neurexin/neuroligins compared to other CAMs, such as ephrins/EphBs, it is likely neurexin and neuroligins do not mediate initial contact, but rather aid in the activation, stabilization and maturation of synapses (Craig and Kang, 2007).

It is also likely that *in vivo*, neuroligins and neurexins cooperate with other CAMs to orchestrate synapse induction and maturation. In support of this idea, the increase in mEPSCs and clustering of synaptic vesicles mediated by neuroligin-1 overexpression was abolished in N-cadherin KO mice cultures (Stan et al., 2010). N-cadherin was also required for the postsynaptic targeting of neuroligin-1 clusters, supporting the idea that neuroligin-1 targeting is independent of PSD-95 (Dresbach et al., 2004; Stan et al., 2010). N-cadherin and neuroligin-1 are likely linked through S-SCAM (Stan et al., 2010). Another study further supported cooperation of

neuroligin-1 and N-cadherin in synapse development, showing that overexpression of N-cadherin in young hippocampal cultures increases neuroligin-1 clustering and spine density, and knockdown of N-cadherin in older cultures decreases the density of neuroligin-1 clusters and synapses (Aiga et al., 2011).

1.3.5 Other Binding Partners for Neurexins

In addition to neuroligins, neurexins bind dystroglycans and neurexophilins (Petrenko et al., 1996; Sugita et al., 2001). Dystroglycans are CAMs that are present at a subset of inhibitory CNS synapses and also neuromuscular junctions, where they help organize the extracellular matrix and mediate clustering of acetylcholine receptors (Grady et al., 2000; Jacobson et al., 2001; Levi et al., 2002). They also bind agrin, Laminin and perlecan. Dystroglycans bind α-neurexins via the second or sixth LNS domain, and bind β-neurexin(-SS4); this binding is calcium-dependent (Sugita et al., 2001). Brain specific KO of dystroglycan in mice results in impaired LTP and muscular dystrophy-like malformations, but normal baseline synaptic transmission (Moore et al., 2002). The functional significance of neurexin binding to dystroglycans in terms of synaptic development or maintenance is still unknown.

Neurexophilin (Nxph) is a secreted glycoprotein that was originally discovered in a purified complex with neurexin1α, and binds via the second LNS domain in a calcium-dependent manner (Missler et al., 1998; Petrenko et al., 1996). Nxph1 and Nxph3 are the two variants that bind neurexin in mice, and both show restricted expression patterns (Beglopoulos et al., 2005; Missler et al., 1998). Knockout mouse studies show that each single neurexophilin mouse has normal brain morphology, and even Nxph1/3 double KO mice are viable. Single Nxph3 mice have increased

startle responses and deficits in motor coordination, suggesting that neurexophilins are important for functions in specific neural circuits (Beglopoulos et al., 2005). However the full significance of neurexophilins binding to α-neurexin has not yet been established.

A new report showed that the extracellular domain of neurexins directly binds to $GABA_A$ receptors with low affinity (Zhang et al., 2010). This interaction suppresses GABAergic synaptic transmission, but did not change the number of GABAergic synapses. This effect was independent of neuroligin-binding, suggesting that presynaptic neurexins may directly influence postsynaptic GABA receptor function (Zhang et al., 2010).

In another very recent report, G-protein coupled receptor CIRL1/Latrophilin-1 (CL1), which is also a receptor for α-latrotoxin, was shown to specifically bind directly to neurexins(-SS4) but not (+SS4) (Boucard et al., 2012). Trans-cellular binding is mediated though the extracellular olfactomedin-like domain of CL1 and is competitive with neuroligin-1. CL1 interacts with SHANK adaptor proteins, suggesting that it may exhibit postsynaptic localization; however, further characterization is needed to determine the functional significance of CL1-neurexin binding (Boucard et al., 2012). Neurexin has also been found to bind to two synapse-inducing molecules in addition to neuroligins: Leucine-rich repeat transmembrane proteins (LRRTMs) and cerebellin1, which will be described below.

1.3.5.1 LRRTMs

LRRTMs were originally discovered in an unbiased screen to identify new synaptic proteins (Linhoff et al., 2009). Shortly thereafter, it was found that neurexins

are their presynaptic binding partners (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010). It appears that LRRTM interaction is also influenced by neurexin splicing, as LRRTM1 and LRRTM2 specifically bind both α- and β-neurexins(-SS4), but not (+SS4), to induce glutamatergic synaptogenesis (Linhoff et al., 2009; Siddiqui et al., 2010). Furthermore, LRRTM1 and 2 are expressed in overlapping patterns with neuroligin-1, and bind β-neurexin(-SS4) at a site overlapping that for neuroligin-1, suggesting competitive binding (Lauren et al., 2003; Siddiqui et al., 2010; Varoqueaux et al., 2006). Like neuroligins, direct aggregation of LRRTM2 induces postsynaptic differentiation and results in recruitment of multiple postsynaptic proteins including GluN1, presumably through LRRTM's PDZ binding domain (Linhoff et al., 2009). LRRTM2 directly binds PSD-95 and also co-immunoprecipitates with GluA1, GluA2 and GluN1; this interaction may facilitate receptor clustering *in vivo* (de Wit et al., 2009; Linhoff et al., 2009). Furthermore, knockdown of LRRTM2 in hippocampal granule cells *in vivo* resulted in a decrease in evoked AMPAR- and NDMAR-mediated transmission (de Wit et al., 2009), but this is controversial as another study showed that both LRRTMs and neuroligin-1 must be knocked down to produce decreases in excitatory transmission (Ko et al., 2011). Last, the distribution of VGlut1 was altered in a laminar-specific pattern in LRRTM1 KO mice (Linhoff et al., 2009). Further studies examining the overlapping expression patterns of LRRTMs, neurexins and neuroligins (including splice variants), as well as combined deletions, will help to elucidate the contribution of each of these proteins in synaptogenesis. For example, an initial study where LRRTM1, LRRTM2 and neuroligin-3 were simultaneously knocked down in neuroligin-1 KO mouse neuron

cultures suggested that neuroligins and LRRTMs are somewhat functionally redundant early in development, but have more divergent roles later in development, with neuroligins being important for NMDAR-mediated synaptic maturation (Soler-Llavina et al., 2011).

1.3.5.2 Cbln1-GluR Delta2

Cerebellin1 precursor protein (Cbln1) is a protein secreted from presynaptic terminals of cerebellar granule cells that binds to the postsynaptic glutamate receptor GluRδ2 on Purkinje cell dendrites (Matsuda et al., 2010). Cbln1 bridges the synaptic cleft to also bind presynaptic neurexins, specifically α– and β-neurexins(+SS4) (Joo et al., 2011; Matsuda and Yuzaki, 2011). The GluRδ2-Cbln1-neurexin(+SS4) complex signals bi-directionally to direct synapse formation between granule cells and Purkinje cells in the cerebellum (Matsuda et al., 2010; Uemura et al., 2010). GluRδ2 contains an intracellular PDZ binding domain, and direct aggregation of GluRδ2 on the postsynaptic side is sufficient to induce clustering of postsynaptic proteins like PSD-95 and SHANK (Matsuda et al., 2010). This complex represents a new mode of action for synaptogenic proteins, by using a secreted protein to link pre- and postsynaptic surface proteins. Both Cbln1 and GluRδ2 KO mice have similar deficits, such as reduced number of parallel fiber-Purkinje synapses, deficits in LTD and mismatches in the length of active zone compared to PSD (Hirai et al., 2005; Kashiwabuchi et al., 1995). Although the Cbln1- GluRδ2 complex selectively mediates synapse development between cerebellar granule cells and Purkinje cells, there are related proteins expressed more widely in the CNS that may have similar functions and overlap with expression of neuroligins and

LRRTMs (Siddiqui and Craig, 2011). In fact, recent studies suggest that cerebellins can also mediate synapse development in transfected cortical neurons (Joo et al., 2011; Yasumura et al., 2011).

1.4 Synaptic Pathways in Neurodevelopmental Disorders

Neurodevelopmental diseases have historically been difficult to study because they appear to be characterized by subtle changes in a subset of neural circuits, rather than a general impairment in all circuits. Furthermore, unlike other disorders like Alzheimer's, pathophysiological correlates have not been found for disorders such as autism and schizophrenia; at this point, diagnosis is made solely based on behavior. Autism spectrum disorders (ASDs) are characterized by impairments in social interactions and communication, as well as restricted patterns of behaviors and interests (DSM-IV-TR, 2000). ASD patients are often hypersensitive to sensory stimulation, and also may exhibit impairments in executive functioning, motor control, and some types of memory (Kootz et al., 1981; Squire and Zola, 1996). Asperger's disorder differs from classical autism in that social and behavioral abnormalities may be milder and there is no marked delay in language development; this may delay the detection of symptoms. However, ASDs are called spectrum disorders as the severity and combination of symptoms varies greatly between children. There are also a few rare disorders that are associated with autism characteristics such as Rett syndrome, fragile X syndrome, tuberous sclerosis and Angelman syndrome, but these are accompanied by more severe phenotypes and other developmental deficits. ASDs are termed

“neurodevelopmental” disorders, as development is normal early in infancy with the onset of symptoms generally occurring from 18 months – 3 years of age. The incidence of ASDs is estimated to be 6 in 1000, with a 4:1 and 8:1 male to female ratio for autism and Asperger’s, respectively (Abrahams and Geschwind, 2008; Fernell and Gillberg, 2010; Levy et al., 2009). Although the prevalence appears to have increased greatly in the last fifty years, this rise could actually be due, at least in part, to better awareness and broader diagnostic criteria to include “higher functioning” cases of ASD, rather than an actual increase in cases. Approximately 10-30% of ASD cases exhibit comorbid epilepsy, prompting some to suggest that imbalances in the E/I ratio may be involved (Gillberg and Billstedt, 2000). Intellectual disability is also common in classical autism, with an approximate prevalence of 60% (Levy et al., 2009).

Thus far, few gross anatomical or morphological changes have been consistently found in ASD. An increase in brain size around the time of symptom onset has been reported in a number of studies, especially in the frontal and temporal lobes and the limbic structure, but is not widely agreed upon (Courchesne et al., 2007; Levy et al., 2009; Schmitz and Rezaie, 2008). Furthermore, this increase in brain size is only observed in about 20% of children, and appears to match (or in some cases even become less than) normal size by adulthood (Courchesne et al., 2001; Courchesne et al., 2007). Functional magnetic resonance imaging studies have suggested that functional connectivity might be perturbed, particularly in areas associated with language and social cognition, while other studies have found hypoactivation in areas related to facial recognition (Levy et al.,

2009). There is currently no cure for ASDs, and treatment focuses mostly on behavioral modifications to deal with social and communication problems, physical therapy, and occupational therapy, as well as pharmacological interventions to treat comorbid disorders such as anxiety, depression and obsessive-compulsive disorders (Levy et al., 2009).

It has recently been hypothesized that schizophrenia may belong on the autism spectrum as well, based on reports of co-incidence of autism and schizophrenia, overlapping clinical phenotypes, similar connectivity deficits, new data showing shared genetic risk factors, and other factors (King and Lord, 2011). Schizophrenia is characterized by “positive” symptoms such as delusions, hallucinations and disorganized speech and behavior, as well as “negative” symptoms such as blunted affect and emotion, lack of motivation, asociality and lack of unprompted normal speech (DSM-IV-TR, 2000). Onset of schizophrenia generally occurs in early adulthood, between the ages of 16 and 30. (Mueser and McGurk, 2004). The overall prevalence of schizophrenia is estimated to be 1% of the population, with men and women being equally affected; however, women tend to have a later age of onset and a milder presentation of symptoms (Mueser and McGurk, 2004). Like ASDs, diagnosis is based on psychiatric evaluation of behavior, as there are currently no reliable biological markers.

Similar to the findings regarding autism, it has been difficult to uncover any gross morphological or structural changes in schizophrenic brains; the most consistent finding from imaging studies is that, in some patients, there are subtle decreases in grey matter, focal alteration of white matter tracts and enlargement of

ventricles (Mueser and McGurk, 2004; Wright et al., 2000). As these types of structural changes typically occur early in development, it has been suggested that schizophrenia can be considered a neurodevelopmental disease. One hypothesis suggests that excessive synaptic pruning accounts for these changes, and onset of symptoms occurs later in life once this pruning has reached a threshold (Innocenti et al., 2003; Keshavan et al., 1994). Functional magnetic resonance imaging and other imaging studies have shown abnormal responses to cognitive and executive function tasks, while neurochemical imaging has implicated aberrant dopaminergic signaling (van Os and Kapur, 2009). The changes in dopamine are validated by the fact that antipsychotic drugs used to treat schizophrenia act by blocking dopamine receptors (Kapur et al., 2006). Non-pharmacological treatment includes cognitive-behavioral therapy and other types of social skills training; there is no cure for schizophrenia. The lack of obvious anatomical correlates for both ASDs and schizophrenia suggests that these are disorders of brain function, rather than structure. As such, much of the current research, especially in autism and related disorders, has focused on trying to find genetic determinants that may underlie these functional changes.

1.4.1 Human Genetic Studies

ASDs are highly genetic, with heritability estimated at 90% based on family and twin studies (Levy et al., 2009). An underlying genetic disorder can be attributed to 10-25% of cases, which includes the rare syndromes such as Rett syndrome and fragile X (Abrahams and Geschwind, 2008). The remaining cases for which a causal factor has not been identified are termed idiopathic ASDs, and genetic and linkage

studies have attempted to find risk factors for these cases. For schizophrenia, heritability is also high, at an estimated 80% (van Os and Kapur, 2009). Previously, genetic research on the common neurodevelopmental and psychiatric disorders was guided by the "common disorder-common variant" model, by which many genetic mutations that are present at a high frequency in the general population would confer a small-to-moderate increase in risk (Cook and Scherer, 2008; Levy et al., 2009). However, genome wide association studies have been unable to produce any reliable results showing such common allele changes that confer even modest risk for any of the main psychiatric disorders (Mitchell, 2011). New technology, such as microarrays, comparative genomic hybridization and high throughput sequencing, has allowed for whole genome linkage and sequencing studies, and the detection of rare genetic changes. This has led to a shift in the field where the "common disorder-rare variant" (also called "multiple rare variants") model suggests that a large number of rare or very rare genetic variants, that are highly penetrant, confer a substantial increase in risk for the disorder (Betancur et al., 2009). Interestingly, a number of the rare variants that have recently been discovered for ASDs are genes for proteins found at synapses, many of which are synaptic adhesion molecules or their interacting partners (Betancur et al., 2009; Bourgeron, 2009).

These variants can be in the form of copy number variants (CNVs), which include deletions and duplications, single nucleotide polymorphisms (SNPs), or other mutations. These genetic changes can either be inherited or *de novo*, but each represents less than 2% of cases individually (Abrahams and Geschwind, 2008). Mutations, CNVs and SNPs have been found in autistic patients in the genes for

neurexin-1 (Bucan et al., 2009; Feng et al., 2006; Szatmari et al., 2007), neurexin-2 (Gauthier et al., 2011), neurexin-3 (Vaags et al., 2012), neuroligin-1 (Glessner et al., 2009), neuroligin-3 (Jamain et al., 2003), and neuroligin-4 (Jamain et al., 2003; Laumonnier et al., 2004; Lawson-Yuen et al., 2008), suggesting this binding pair may have a central role in some cases of ASD. Other genes for synaptic proteins implicated in ASDs and/or intellectual disability include the synaptogenic protein LRRTM-3 (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010; Sousa et al., 2010); the synaptogenic protein SynCAM (Biederer et al., 2002; Zhiling et al., 2008); the synaptogenic protein SALM5 (Mitchell, 2011; Xu et al., 2009), the CAMs contactins-3 and -4 (Karagogeos, 2003; Roohi et al., 2009); the contactin-associated protein-2, which is similar in structure to neurexins (Bakkaloglu et al., 2008; Pillai et al., 2007); certain members of the cadherin and protocadherin families of “adhesive” CAMs (Bhalla et al., 2008; Marshall et al., 2008; Morrow et al., 2008); L1 CAM, which mediates neuronal migration and synaptic targeting (Kenwrick et al., 2000); CASK, which directly binds neurexins to mediate calcium channel function (Froyen et al., 2007; Hata et al., 1996; Najm et al., 2008); Mint2, the adaptor protein which binds CASK and neurexins (Guilmatre et al., 2009), PSD-95, the major excitatory scaffolding protein, and its relative PSD-93 (Mitchell, 2011; Peca et al., 2011b; Walsh et al., 2008); the SHANK2 and SHANK3 scaffolding proteins, which indirectly bind neuroligins via PSD-95 (Berkel et al., 2010; Durand et al., 2007; Moessner et al., 2007; Peca et al., 2011a; Sheng and Hoogenraad, 2007); as well as other synapse-associated proteins (Abrahams and Geschwind, 2008; Aldinger et al., 2011; Betancur et al., 2009; Mitchell, 2011; Peca et al., 2011b; Pinto et al., 2010). It

is important to note that there is often more than one type of mutation identified for each of these genes, and each mutation is itself very rare. For example, there have been seven different point mutations, two translocations and four different large scale deletions reported for neurexin-1 and two frameshift mutations, five missense mutations and three internal deletions reported for neuroligin-4 (Sudhof, 2008). In other cases, synaptic genes are affected by large scale deletions, such as the five different CNVs which encompass the neuroligin-4 gene locus (Sudhof, 2008).

In addition, genes for neurexin-1, neurexin-2, LRRTM-1, SALM5, PSD-93, PSD-95, SHANK3, contactin-5, contactin-associated protein-1 and -2, GluRδ2, Mint2, as well as a number of synaptic vesicle release machinery proteins, have all been linked to schizophrenia (Fallin et al., 2005; Francks et al., 2007; Gauthier et al., 2010; Gauthier et al., 2011; Guilmatre et al., 2009; Kirov et al., 2008; Peca et al., 2011b; Pickard, 2011; Waites and Garner, 2011; Walsh et al., 2008; Xu et al., 2009). This suggests there may be similar underlying synaptic causes and/or risk factors for schizophrenia and ASDs, which may manifest differently depending on other environmental and genetic factors influencing each individual case. It is currently unclear exactly how mutations in synaptic genes, which may affect synapse formation, pruning, maintenance and/or plasticity, lead to the varying and complex behavioral characteristics of ASDs and schizophrenia. It is interesting to note, however, that the onset of ASDs coincides with the time of peak postnatal synapse formation and maturation in humans (Turrigiano and Nelson, 2004). New studies, which model some of these disease-associated mutations in mice, are beginning to shed light on this question.

1.4.2 Animal Models

Using mouse models to study neurodevelopmental disorders is not an easy task. As previously mentioned, diagnosis for these disorders is currently based on behaviors; many of these, such as the obsessive interests in ASDs or the hallucinations in schizophrenia, are near impossible to model in mice. However, many behaviors can be modeled in mice, such as anxiety and socialization. Another problem is that the onset of ASDs occurs quite early, and mice pups at the corresponding developmental stage do not have many behaviors that can be reliably measured; thus, behavioral tests are generally done with more mature mice. The first breakthroughs in modeling ASDs came from studies of so-called “syndromic autism,” where a diagnosis of autism is secondary to other more severe developmental deficits. These disorders, including Rett syndrome, fragile X syndrome and Angelman syndrome, are easier to model because a causal genetic mutation is identified and well characterized in each case.

Rett syndrome is an X-linked disorder affecting 1 in 10,000 girls in which development progresses normally until 6 to 18 months, followed by an onset of developmental regression. Symptoms include decreased brain growth, loss of purposeful hand skills and/or stereotyped hand movements, breathing abnormalities, loss of motor coordination, epilepsy, autism, and shortened lifespan (DSM-IV-TR, 2000). Most cases of Rett syndrome are caused by a mutation in the gene for methyl CpG binding protein 2 (MeCP2); MeCP2 binds to methylated DNA to repress or activate gene expression (Amir et al., 1999). MeCP2 KO mice or mice expressing a truncated version of MeCP2 (which is found in some Rett’s patients) have similar

neurological phenotypes to human patients, such as onset of symptoms at 5-6 weeks of age, motor coordination defects, reduced social interaction, increased anxiety, shortened lifespan, deficits in LTP and impaired learning and memory, as well as decreased number of synaptic connections (Chen et al., 2001; Moretti et al., 2005; Moretti et al., 2006; Shahbazian et al., 2002). Studies with postnatal, forebrain-specific loss of MeCP2 show that it plays a role in adult neuron function in addition to its roles in development, while replacement of MeCP2 in adult mice can rescue some, but not all, of the behavioral and neurological deficits, suggesting that some features of Rett's are reversible (Chen et al., 2001; Guy et al., 2007; Jugloff et al., 2008). Furthermore, treatment with insulin-like growth factor, which plays a role in stimulating synaptic maturation, ameliorated many of the symptoms of the Rett-like phenotype in juvenile MeCP2 KO mice, including locomotion and respiration problems, decreased brain weight, decreased cortical spine density and shortened lifespan (Robertson and Feng, 2011; Tropea et al., 2009). The results were so positive in this model system that phase I and II clinical trials are currently underway to use insulin-like growth factor to treat children with Rett syndrome (LeBlanc and Fagiolini, 2011).

Fragile X syndrome predominantly affects males and is characterized by mental retardation, hyperactivity, facial dysmorphology, anxiety, motor problems, autistic-like behaviors and, in many cases, epilepsy (Garber et al., 2008). Fragile X is most commonly caused by a mutation in the promoter of the gene for Fragile X mental retardation 1 protein (FMRP), which results in transcriptional silencing of the gene and reduction in FMRP protein levels (Verkerk et al., 1991). FMRP is an

mRNA binding protein, regulating the translation and mRNA transport of many synaptic proteins. Postmortem analysis of fragile X brains showed an increased number of immature-looking spines in excitatory cortical neurons, which is mirrored in FMRP KO mice (Hinton et al., 1991; Irwin et al., 2000). These mice also display a range of behavioral abnormalities that may represent fragile X, such as increased seizures, decreased spatial learning and object recognition, deficits in social interaction and increased social anxiety (Robertson and Feng, 2011). Enhanced metabotropic glutamate receptor (mGluR)-dependent LTD is also observed in FMRP KO mice; this led to the "mGluR theory" for fragile X in which reduction in FMRP levels releases the negative regulation of mGluR-dependent LTD, which in turn leads to exaggerated LTD and net loss of synapses (Bear et al., 2004). In support of this hypothesis, genetic knockdown or pharmacological inhibition of mGluRs improve many of the phenotypes of FMRP protein reduction, including the increase in seizures and the decrease in spine density (LeBlanc and Fagiolini, 2011; Robertson and Feng, 2011). A number of fragile X clinical trials are currently underway for various mGluR antagonists (LeBlanc and Fagiolini, 2011).

Angelman syndrome is characterized by normal development during the first year of life, followed by the onset of progressive mental retardation, motor dysfunction, speech impairment and a high rate of autism (Clayton-Smith and Laan, 2003). It is caused by maternal deletion of chromosome 15q11-13, or by more specific deletions in the gene for E3 ubiquitin ligase (Ube3a), which is found in this region. Inactivation of the maternal copy of Ube3a in mice results in enhanced seizures, deficits in motor coordination, reduced spatial learning, deficits in LTP and

deficits in experience-dependent maturation of excitatory circuits (Jiang et al., 1998; Weeber et al., 2003; Yashiro et al., 2009). The 15q11-13 region also contains several genes for $GABA_A$ receptor subunits, among which is the Gabrb3 gene for the $GABA_AR$ β3 subunit. KO mice for Gabrb3 also exhibit an Angelman syndrome-like phenotype, including hyperactivity, increased seizures, deficits in motor coordination, impaired social interaction, and impaired learning and memory (DeLorey et al., 1998; DeLorey et al., 2008; Homanics et al., 1997).

These cases of modeling syndromic autism, although somewhat confounded by other comorbid deficits, help to shed light on the molecular mechanisms that may underlie autism; in the case of Rett syndrome and fragile X, these studies are also leading to new therapeutic avenues. The case is not as clear-cut for many of the newly discovered rare variants associated with ASD. Some of the mutations are found in intronic regions, making them both difficult to model in culture or in animal models, and difficult to predict how these might affect transcription, translation and/or protein function to ultimately mediate a disease phenotype. Nevertheless, some progress has been made in modeling exonic disease mutations for a number of synaptic proteins, especially SHANK3 and neuroligin-3 and -4.

CNVs and SNPs in SHANK3 are associated with ASDs, and deletion of the SHANK3 gene is the causative agent in 22q13.3 deletion syndrome, which is characterized by developmental delay, minor dysmorphic features and autistic behaviors (Bonaglia et al., 2006; Cusmano-Ozog et al., 2007). Three recent studies have shown autistic behaviors and synaptic deficits in SHANK3 mutant mice. One group created mice in which the SHANK3 gene was disrupted on one chromosome

in order to model haploinsufficiency observed in humans. These SHANK3 heterozygous mice had decreased AMPAR-mediated basal synaptic transmission, reduced numbers of GluA1 puncta and impaired LTP, as well as social impairments and deficits in vocalization (Bozdagi et al., 2010). In mutant mice where exons 4-9 were deleted, abnormal social behaviors and communication patterns, repetitive behaviors, and deficits in learning and memory were observed (Wang et al., 2011). These mice also displayed reduced levels of some postsynaptic proteins, subtle alterations in dendritic spine morphology, and deficiencies in LTP (Wang et al., 2011). A third study generated two different SHANK3 mutant mice based on deletions found in humans: one which disrupted one of three main SHANK3 isoforms, and a second which disrupted two SHANK3 isoforms and drastically reduced the levels of the third. Although both homozygous mice showed behavioral deficits, the mutant mice with the more severe disruption showed the more pronounced phenotype. These mice displayed self-injurious repetitive grooming and deficits in social interaction, as well as morphological and functional perturbations of synapses in the striatum and cortico-striatum circuits (Peca et al., 2011a). These three studies highlight an important point: although all three types of mutant mice displayed behavioral abnormalities that are reminiscent of ASDs, their phenotypes were not exactly identical. This may reflect the subtle difference in SHANK3 mutations in each of these mice, and suggests possible mechanisms by which an autism “spectrum” of disorders may arise even from different mutations in the same gene.

Mutations in neuroligin-3 and -4 have also been the subject of recent investigation. Both of these genes are on the X-chromosome in humans; X-linked mutations may be connected to the higher incidence of ASDs in males. In neuroligin-3, a missense mutation (R451C) found in autism was directly modeled in mice. These “R451C” mice show normal motor and anxiety behaviors, a moderate impairment in social interaction, and an increase in spatial learning capability (Tabuchi et al., 2007). The increase in learning was surprising considering that human patients with this mutation have learning disabilities (Jamain et al., 2003). Electrophysiological examination revealed in increase in inhibitory transmission in the somatosensory cortex, as well as an increase in GABAergic synapse density, resulting in a shift in the E/I ratio towards inhibition. Interestingly, analogous behavioral and functional phenotypes were not observed in a neuroligin-3 KO mouse, suggesting the R451C is a gain-of-function mutation, despite the fact that mutant protein is partially retained inside the cell (Chih et al., 2004; Comoletti et al., 2004; Tabuchi et al., 2007). However, another study using R451C knock-in mice did not find any deficits in social interactions (Chadman et al., 2008). Other studies have mimicked a stop-codon mutation in neuroligin-4 by generating a neuroligin-4 KO mouse. These mice showed clear evidence of a loss-of-function mutation in mice. These mice have deficiencies in reciprocal social interactions, and decreases in vocalization (Jamain et al., 2008). Interestingly, recent crystallization and structure-mapping studies have shown that both neuroligin-3 and neuroligin-4 ASD-associated mutations map to sites distal from the neurexin-binding face of neuroligins,

suggesting that these mutations may interfere with functions of neuroligins that are independent of their binding to neurexins (Koehnke et al., 2008)

An emerging theme in models of ASD seems to be disruptions in the E/I balance. Closer inspection of mouse models for syndromic autism also suggest imbalances in the E/I ratio (LeBlanc and Fagiolini, 2011). It is also important to note that straight KO or overexpressing mice have been generated for some of the families of synaptic CAMs associated with autism, and some of these mice show autism-like behaviors and/or changes in E/I balance as well, such as the neuroligin-1 and -2 overexpressing and KO mice (see Sections 1.2 and 1.3.4). More thorough characterization of behavioral phenotypes of hetero- or homozygous KO mice that already exist for synaptic proteins associated with neurodevelopmental disorders, as well as directly modeling disease mutations in mice, is merited to further elucidate how such varied mutations in a number of synaptic proteins can contribute to the autism phenotype at the molecular and behavioral level.

1.5 Calsyntenins

In this thesis, evidence will be provided for a new role for the protein calsyntenin-3 in synapse development (Chapter 2). The calsyntenin family has not been studied extensively, and most work has focused on the calsyntenin-1 family member. The following comprises a brief review of the current body of published work on this family of proteins.

1.5.1 Structure and Expression Patterns of Calsyntenins

Calsyntenin-1 was first discovered in a screen to identify secreted proteins in synapse-forming chicken spinal cord neurons (Vogt et al., 2001), which was driven by the discovery that serine proteases had been implicated in learning and memory (Huang et al., 1996; Madani et al., 1999; Qian et al., 1993). Purification and cDNA sequencing revealed a protein with a large extracellular N-terminal domain, a single transmembrane domain, and a cytoplasmic C-terminal domain, making it a type I transmembrane protein (Vogt et al., 2001). A follow-up study reported the cloning of two other family members in mouse, calsyntenin-2 and calsyntenin-3 (Hintsch et al., 2002). All three calsyntenins have two cadherin-like repeats and an LNS-like domain in the extracellular region, followed by a single-pass transmembrane domain and a short cytoplasmic tail. The C-terminal region contains a putative calcium binding site; in calsyntenin-1 this site has been shown to have high (~0.5 μM) and low affinity Ca^{2+}-binding capacities; however, the potential physiological significance of calcium binding by calsyntenins has not been investigated further (Vogt et al., 2001). Furthermore, the acidic residues that comprise the presumed Ca^{2+} binding site in calsyntenin-1 are not well conserved in calsyntenin-2 and -3, suggesting that the binding affinity would be lower for calsyntenin-2, while calsyntenin-3 is unlikely to bind calcium at all (Hintsch et al., 2002). This diversity is in contrast to the highly conserved extracellular domains of calsyntenins. Calsyntenins seem to be highly conserved across species: humans and rats both have three corresponding calsyntenins, while single calsyntenin genes were found in *Drosophila* and *C. elegans* (Hintsch et al., 2002; Vogt et al., 2001).

Northern blot analysis of murine tissues revealed that all three calsyntenins are predominantly expressed in the brain, with calsyntenin-2 and -3 being exclusively expressed here (Hintsch et al., 2002; Vogt et al., 2001). Calsyntenin-2 is also expressed at a much lower level than calsyntenin-1 and -3 in the brain (Hintsch et al., 2002). Low levels of calsyntenin-1 were also detected in kidney, lung, skeletal muscle, heart and testis (Hintsch et al., 2002). Expression of calsyntenins in the brain was also found in human tissues (Hintsch et al., 2002). Other tissues showing expression in humans included the heart, skeletal muscle, kidney and placenta for calsyntenin-1, and the kidney, heart, skeletal muscle, liver, placenta, pancreas and lung for calsyntenin-3 (Hintsch et al., 2002). Using light and electron microscopy, as well as subcellular fractionation, all three calsyntenins were found at the postsynaptic membrane, predominantly at asymmetric (excitatory) synapses, although they were also found at lower levels in internal membranous organelles, the endoplasmic reticulum, and the Golgi apparatus (Hintsch et al., 2002; Vogt et al., 2001). Further *in situ* hybridizations in adult mouse brain revealed distinct neuronal expression patterns for the three calsyntenins throughout the brain, with calsyntenin-1 having the most uniform expression throughout most neuron subpopulations, and calsyntenin-2 and -3 being much more variable (Hintsch et al., 2002). The overall highest expression for calsyntenin-2 and -3 was in putative GABAergic neurons in the cerebral and cerebellar cortex (Hintsch et al., 2002). In the hippocampus, calsyntenin-1 is expressed at high levels in CA1-3 regions, and also shows moderate expression in dentate gyrus granule cells. Calsyntenin-2 expression is high in the CA2-CA3 region, and in some scattered interneurons in the pyramidal

cell layer of CA1, and only moderately in other pyramidal cells of the CA1 region; it is expressed at low-moderate levels in dentate gyrus granule cells. Strong expression of calsyntenin-3 was also observed in pyramidal neurons in the CA2-CA3 region, and in interneurons in the pyramidal layer of the CA1 region, while only moderate levels were detected in the CA1 region; calsyntenin-3 was not detected in the dentate gyrus except for a small population of interneurons in the hilus (Hintsch et al., 2002).

As mentioned, the N-terminal portion of calsyntenin-1 was originally isolated as a secreted protein, suggesting proteolytic cleavage in the synaptic cleft (Vogt et al., 2001). Further analysis revealed that the C-terminal stump resulting from cleavage is internalized and accumulates in the spine apparatus on spine synapses and the subsynaptic membranes of shaft synapses (Vogt et al., 2001). Interestingly, another group independently discovered calsyntenin-1 in a yeast-two-hybrid screen to find proteins that interact with the X11-like (X11L)/Mint2 protein, and termed it Alcadein (**Al**zheimer-related **cad**herin-like prot**ein**) (Araki et al., 2003); however, for the purposes of this thesis, I will refer to this family as calsyntenins as this was the originally reported nomenclature. Meanwhile, in a follow-up study to the first two calsyntenin studies, a yeast-two-hybrid screen with calsyntenin-1 C-terminal as bait identified a direct interaction between calsyntenin-1 and the light chain of kinesin-1 (KLC-1), a motor protein (Konecna et al., 2006). Thus, two somewhat divergent streams of research have emerged to characterize these two separate interactions, and seem to suggest a role for calsyntenins in both Alzheimer's disease (Section 1.5.2) and as cargo docking proteins (Section 1.5.3).

1.5.2 Calsyntenins in Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease which is clinically characterized by gradual loss of memory and cognitive disturbances (DSM-IV-TR, 2000). Definitive diagnosis of AD can only be made via postmortem autopsy, and is based on the presence of β-amyloid plaques and neurofibrillary tangles in the brain (DSM-IV-TR, 2000). β-amyloid plaques are composed of amyloid β-protein (Aβ) aggregates, which are themselves products of the regulated cleavage of the APP protein. Production and accumulation of Aβ are initial steps in the pathogenesis of AD. APP is cleaved in two main pathways: amyloidogenic and non-amyloidogenic. In the non-amyloidogenic pathway, APP is initially cleaved in the extracellular domain by α-secretase (ADAM10 or ADAM17) to yield a soluble N-terminal fragment (sAPPα), and a C-terminal fragment (CTFα); subsequent intramembrane cleavage of CTFα by the γ-secretase complex produces secreted p3 peptide and an intracellular domain (AICD). In the amyloidogenic pathway, β-secretase (BACE) initially cleaves the extracellular domain of APP at a site slightly closer to the N-terminus than α-secretase to produce sAPPβ and CTFβ; the γ-secretase complex then cleaves the CTFβ to produce an intracellular domain and Aβ peptide, which is slightly longer than p3 peptide (Price et al., 1998; Selkoe, 2001). The γ-secretase complex can cleave the CTF at a few different sites, giving rise to different lengths of Aβ, with Aβ42 being the more fibrillogenic and neurotoxic. The Aβ42/40 ratio is sometimes used as a measure of drug efficacy in patients or in experiments (Selkoe, 2001). Thus, the regulation of APP cleavage plays an important role in the pathogenesis of AD.

X11L/Mint2 was originally identified as an intracellular binding partner for APP, and binding results in the suppression of Aβ production (Tomita et al., 1999). In an effort to find other binding partners for Mint2 that may regulate the cleavage of APP, a yeast-two-hybrid screen was done using Mint2 as bait. A novel interacting cDNA was discovered and entitled Alcadeinα1, which corresponds to the previously reported calsyntenin-1. The other two calsyntenins were termed Alcadeinβ and Alcadeinγ and correspond to calsyntenin-3 and calsyntenin-2, respectively (Araki et al., 2003). Further analysis showed that both calsyntenin-1 and -3 interact with the phosphotyrosine interaction domain of Mint2 via a conserved sequence in the C-terminal domain; calsyntenin-2 was not tested (Araki et al., 2003). Binding to Mint2 was found to be cooperative, such that calsyntenin-1 and APP intracellular domains bind Mint2 simultaneously to form a tripartite complex; the formation of this complex enhances the stabilization of APP by Mint2 and suppresses cleavage by γ-secretase, resulting in decreased Aβ production (Araki et al., 2003). This study also found that in human AD brain samples, calsyntenin-1, APP and Aβ were co-localized in dystrophic neurites in senile plaques.

A subsequent study found that after the initial extracellular cleavage, calsyntenin-1, -2 and -3, like APP, are also subject to γ-secretase cleavage (Araki et al., 2004). Further analysis showed that processing of calsyntenin-1 proceeds in a similar fashion to APP, with the putative primary cleavage site being close to the transmembrane domain, and secondary cleavage by γ-secretase leading to the formation of Aβ-like peptides (termed β-Alcα) (Araki et al., 2004). The processing of calsyntenins are also suppressed when they form the calsyntenin-Mint2-APP

tripartite complex, suggesting that binding of both proteins to Mint2/X11L may block access of the presenilin component of γ-secretase to the transmembrane domains of calsyntenin-1 and APP. This study also found that the intracellular domain of calsyntenin-1 (ICD) generated from γ-secretase cleavage can translocate to the nucleus and suppresses FE65-dependent gene transactivation by APP, likely by competing with APP for binding, suggesting that calsyntenins can influence gene transcription as well (Figure 1.5) (Araki et al., 2004).

Further characterization of the cleavage of calsyntenins revealed that the α-secretases ADAM10 and ADAM17 are both able to mediate the primary juxtamembrane cleavage of all three calsyntenins, just like APP (Hata et al., 2009). This study also showed that BACE is likely not involved in the primary cleavage of calsyntenins, thus the previously termed "β-Alc" peptides are in fact more akin to APP p3 peptides and were thus renamed "p3-Alc" peptides. Recovery of p3-Alc proteins and analysis using mass spectroscopy allowed for the precise mapping of primary and secondary cleavage sites in all three calsyntenins (Hata et al., 2009). Using a presenilin-1 mutant that is linked to familial AD, the authors also showed that this mutant modulates the ratio of p3-Alc peptide length in a similar manner as it modulates the length of Aβ peptides (the 42/40 ratio), suggesting that these processes may be correlated; however, it is important to note that p3-Alc peptides are not prone to aggregation. Furthermore, p3-Alc fragments were found in human AD cerebrospinal fluid samples. This fact, combined with their covariance with Aβ42/40 ratios, suggests they may be valuable as a diagnostic tool for γ-secretase function (Hata et al., 2009). In fact, a study with human subjects showed that

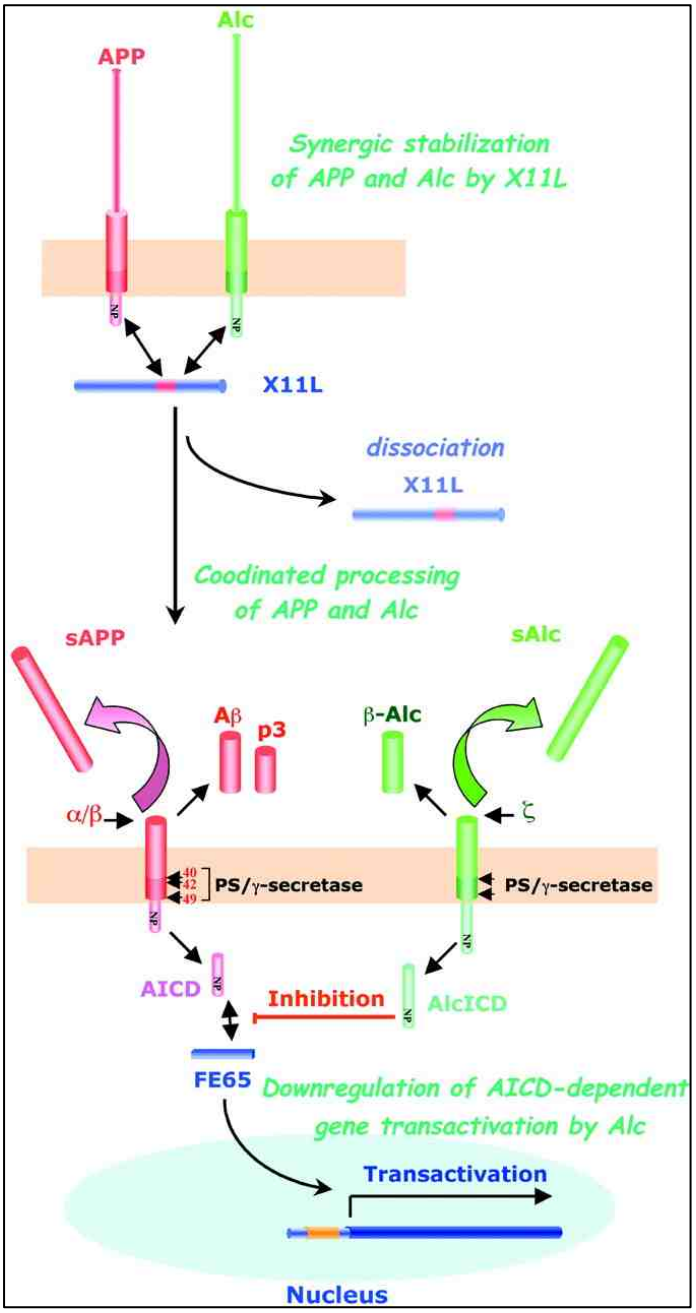

Figure 1.5: Coordinated Processing of APP and Calsyntenins
(TOP) APP and Alc (aka calsyntenin) are stabilized by formation of a tripartite complex with X11L/Mint2. Dissociation of X11L leaves APP and Alc vulnerable to coordinated proteolytic processing (MIDDLE). Primary cleavage results in the secretion of large ectodomains (sAPP or sAlc) and C-terminal fragments (CTFs). Subsequent cleavage by presenilin (PS)/γ-secretase results in the production of Aβ or p3 peptides in the case of APP, or β-Alc peptides in the case of calsyntenins. (BOTTOM) The intracellular domains (ICD) of APP and calsyntenins, which are liberated from the membrane via γ-secretase cleavage, can interact with the nuclear adaptor protein FE65 in the cytoplasm or the nucleus to influence gene transactivation. Binding to FE65 appears to be competitive, thus AlcICD may inhibit AICD binding to FE65 and subsequent activation of transcription. Figure reproduced with permission from Araki et al., 2004, Journal of Biological Chemistry, © the American Society for Biochemistry and Molecular Biology.

increases in Aβ40 levels were found in some patients, and were correlated with p3-Alcα found in plasma (Konno et al., 2011). These authors developed a simple enzyme-linked immunosorbent assay to detect p3-Alcα, and suggest that quantification of p3-Alcα may be a useful biomarker for AD.

Another recent study compared proteins in cerebrospinal fluid of carriers of familial AD mutations (most of whom were still asymptomatic) to those of related non-carriers, and found that the levels of calsyntenin-3 were decreased 1.32 times in carriers, but not non-carriers (Ringman et al., 2012). However, due to the fact that this study linked protein fragments found in cerebrospinal fluid and identified by mass spectroscopy to full length sequences, it is not known which cleavage product(s) of calsyntenin-3 this figure represents. Furthermore, in this same study, the levels of APP were increased, suggesting that, at least at this presymptomatic stage of the disease, APP and calsyntenin-3 levels may not be correlated (Ringman et al., 2012). Differences could also be due to the cohort of patients or the underlying disease cause, as calsyntenin processing has only been linked to presenilin/γ-secretase dysfunction, while the study with familial AD carriers included patients with either presenilin or APP mutations.

Further conflicting results came from a very recent study in which cultured cortical rat neurons treated with Aβ42 peptides resulted in an upregulation of calsyntenin-3, downregulation of calsyntenin-2, and no change in calsyntenin-1 mRNA levels (Uchida et al., 2011). This increase in calsyntenin-3 mRNA was accompanied by increased protein expression with oligomergic, but not fibrillar

Aβ; calsyntenin-3 (but not -1 or -2) expression was also increased in the brains of APP mutant mice (APP_{SW}, Tg2576), which are used as a model for AD. Thus, it is possible that APP and/or Aβ levels differentially regulate calsyntenin family members. Double staining in APP mutant mouse brains for calsyntenin-3 with APP and tau showed that calsyntenin-3 distribution was similar to APP, suggesting that it may be accumulated in dystrophic neurites (Uchida et al., 2011). Interestingly, overexpression of calsyntenin-3 in cortical neurons also increased vulnerability to serum withdrawal and subsequent cell death. The authors suggest that perhaps soluble Aβ oligomers upregulate calsyntenin-3 expression, which would result in synaptic degeneration and cell death (Uchida et al., 2011). However, the mechanism by which this might be accomplished is still unknown.

1.5.3 Calsyntenins as Cargo Docking Proteins

Separate to the possible coordinated metabolism and/or co-regulation of calsyntenins and APPs, a mounting body of evidence has also connected calsyntenins, particularly calsyntenin-1, to vesicle transport via kinesins. Kinesins are motor proteins that transport cargo along microtubules towards the plus end. Kinesin-1 is composed of two light chains (KLC) and two heavy chains (KHC) (Hirokawa et al., 1989a). In an initial study, a direct interaction with KLC1 was discovered in a yeast-two-hybrid screen using the C-terminal fragment of calsyntenin-1 as bait (Konecna et al., 2006). Further analysis showed that calsyntenin-2 also bound to KLC1, while binding to calsyntenin-3 was weaker. Binding between calsyntenin-1 and KLC1 is mediated by the tetratricopeptide repeats (TPR) in KLC1 and by two “WDDS” motifs in calsyntenin-1, termed KLC1-

binding segment 1 and 2 (KBS1, KBS2). The conservation of only KBS1 and not KBS2 in calsyntenin-3 explains the weaker binding to KLC1. Immunocytochemistry of cultured neurons showed that calsyntenin-1 was localized to axonal growth cones and was associated with a population of vesicles that are distinct from synaptic vesicle precursors (Konecna et al., 2006). Furthermore, mutation of the KBS 1 and/or 2 disrupted the anterograde transport of calsyntenin-1-positive vesicles (Konecna et al., 2006).

A second independent study confirmed direct interaction between calsyntenin-1 and KLC and fast anterograde transport of calsyntenin-1 cargo vesicles via microtubules (Araki et al., 2007). Interestingly, calsyntenin-1 appears to compete with another cargo docking protein, JIP1b, for docking to kinesin-1; JIP1b may act as a cargo protein for APP-containing vesicles. This study found that calsyntenin-1 was transported, for the most part, independently of APP, and overexpression of the intracellular domain of calsyntenin-1 suppressed the transport of APP-containing vesicles and lead to an increased generation of Aβ (Araki et al., 2007). Interestingly, although full length calsyntenin-1 can also inhibit APP transport, it also competitively inhibits APP cleavage resulting in an apparent decrease in Aβ. In addition, overexpression of the single calsyntenin in *Drosophila* larvae resulted in locomotor defects and an accumulation of APPL (the *Drosophila* homolog for APP)-containing vesicles in axons, suggesting that calsyntenin-1 can regulate APP transport and motor neuron function *in vivo* (Araki et al., 2007).

However, independent transport of APP and calsyntenin-1 was not wholly supported by a subsequent study, which showed that APP, calsyntenin-1 and KLC1

were tightly associated in the Golgi apparatus, and that APP and calsyntenin-1 were mostly co-localized in organelles in fibroblasts (Ludwig et al., 2009). Partial co-localization of APP and calsyntenin-1 in the Golgi, dendrites and axons was confirmed in hippocampal neuron cultures and in mouse brains, where they were mostly located in vesicular or tubular structures. Lastly, the generation of APP CTFs was increased when calsyntenin-1 was knocked down in cortical cultures, but the level of full length APP did not change significantly (Ludwig et al., 2009). One explanation for the discrepancies between these two studies may be that there are three populations of carriers containing (1) APP and calsyntenin-1, (2) APP only and (3) calsyntenin-1 only. Furthermore, the effects on co-transport and the coordinated or competitive effects on proteolysis are difficult to dissect, and also likely depend on cell type and overexpression / knockdown level (Ludwig et al., 2009).

A new study suggests that binding of KLC1 to calsyntenin-1 is regulated by extracellular-signal-regulated kinase (ERK)-mediated phosphorylation of a specific residue in KLC1 (Vagnoni et al., 2011). Phosphorylation reduced binding, while inhibition of ERK increased binding of calsyntenin-1 to KLC1. Mutation of this residue to mimic permanent phosphorylation reduced the co-localization of calsyntenin-1 and KLC1, and also reduced anterograde transport while increasing retrograde transport of calsyntenin-1-containing organelles in rat cortical neurons; a mutation to prevent phosphorylation had the opposite effects (Vagnoni et al., 2011). How phosphorylation is regulated is therefore expected to play an important role in the transport and localization of calsyntenin-1.

The composition of calsyntenin-1 growth cone organelles was recently investigated, and revealed that proteins were mainly vesicle transport proteins (including SNAPs, syntaxins, Rab GTPases, synaptotagmin 1, and synapsin-3), recycling membrane proteins (including APP and L1-CAM) and membrane transporter proteins (including Na^{+}/Ca^{2+} -exchanger 1, Na^{+}/K^{2+} -transporting ATPase chains, and vacuolar ATP synthase subunits), as well as a fraction of miscellaneous proteins (including SHANK1, myelin basic protein S and contactin 1) (Steuble et al., 2010). Further inspection showed that two distinct populations of cargos exist: one contains early-endosome proteins, as well as APP, and the second contains recycling-endosome proteins. Interestingly, in the population of early-endosome transport organelles, the subpopulation which contain APP also contain full length calsyntenin-1, while the subpopulation without APP are enriched for cleaved calsyntenin-1 (Steuble et al., 2010). This study suggests that calsyntenin-1 may influence developmental processes like axon growth and pathfinding and/or may help maintain neuronal polarity through acting as a kinesin-1 docking protein. Clearly, more work is needed to clearly define the roles of calsyntenin-1 in the trafficking and processing of APP, as well as the trafficking of other synaptic components. It is also unknown whether calsyntenin-2 and -3 are able to function in similar ways to mediate trafficking, or whether they play distinct roles.

1.5.4 Calsyntenins in Learning and Memory

Is it clear that most research up until this point has focused on calsyntenin-1. However, three studies recently linked a SNP in calsyntenin-2 with human episodic memory performance (Jacobsen et al., 2009; Papassotiropoulos et al., 2006;

Preuschhof et al., 2010). The SNP consists of a common T → C substitution and is located in the first intron of calsyntenin-2, with the "C" allele conferring enhanced performance on memory tasks; this enhancement was associated with increased activity in the hippocampus (Jacobsen et al., 2009). The SNP in calsyntenin-2 was associated with another SNP in the gene for KIBRA (kidney and brain expressed protein) in these memory tasks, and the presence of both SNPs together boosted memory performance further (Preuschhof et al., 2010). However, as both of these SNPs are located in introns, it is unclear how they might affect memory performance.

Interestingly, the single *C. elegans* ortholog of the calsyntenin family, CASY-1, was isolated in a screen to identify worms that were deficient in a learning task (Ikeda et al., 2008). The isolated mutant carried a single missense mutation (*pe401*; E → K) in the LNS domain. CASY-1 shares the closest homology to vertebrate calsyntenin-2 (Hoerndli et al., 2009). Further characterization showed that CASY-1 is needed in mature adult circuits to mediate roles in learning and memory, not just during development, even though it was expressed in the nervous system from the embryonic stage. Unlike studies in vertebrates showing postsynaptic localization, CASY-1 is expressed in cell bodies of neurons in *C. elegans*, both in intracellular membranes and at the plasma membrane; however, ectodomain cleavage was still detected for CASY-1 (Ikeda et al., 2008). Learning defects could be rescued using only the ectodomain of CASY-1, even a version lacking the transmembrane domain, suggesting that cleavage and release of the extracellular domain may modulate learning via autocrine or paracrine signalling. Further domain analysis showed that

the LNS domain is critical for learning, while the cadherin domains may play an accessory role (Ikeda et al., 2008). A role for CASY-1 in learning and memory was further confirmed in a subsequent study showing multiple sensory learning defects, but no sensory impairment, in *C. elegans* mutants which express a truncated version of CASY-1 lacking most of the extracellular and the entire intracellular domain (Hoerndli et al., 2009). Remarkably, these learning defects were rescued by expression of human calsyntenin-2. Increasing gene dosage of GLR-1, which is an AMPA-like *C. elegans* glutamate receptor, was also able to compensate for the behavioral deficits, suggesting that CASY-1 may regulate GLR-1 signaling in learning and memory (Hoerndli et al., 2009). Further studies are needed to confirm if calsyntenin-2 plays similar roles in learning and memory in vertebrate model systems, and if so, what mechanisms may be involved.

1.6 MDGAs

In Chapter 3, I will show that the MAM domain containing glycosylphosphatidylinositol anchor (MDGA) family of proteins is involved in synapse modification. Previous work on MDGAs has focused on their roles in neuronal migration and layer-specific patterning during development; these studies will be reviewed briefly below.

1.6.1 Structure and Expression Patterns of MDGAs

There are two MDGA family members, MDGA1 and MDGA2. MDGA1 was originally discovered in a differential display PCR screen to search for genes involved in rat basilar pons development (Litwack et al., 2004). Subsequent cloning

revealed a protein of 956 amino acids consisting of six immunoglobulin (Ig) repeats, a fibronectin type III-like (FNIII) domain, and a meprin, A5 protein, receptor protein tyrosine phosphatase mu (MAM) domain, followed by a GPI-anchoring site. Homology searches revealed another highly related rat gene with very similar domain structure to MDGA1: six Ig repeats, a highly conserved MAM domain and a GPI-anchoring site, which was termed MDGA2 (Litwack et al., 2004). However, the amino acid stretch corresponding to MDGA1's FNIII-like domain does not bear similarity to fibronectin in MDGA2. Both MDGA1 and 2 are conserved in humans.

Northern blot analysis and *in situ* hybridization revealed that MDGAs are brain specific, with little expression detected outside the central and peripheral nervous systems (Litwack et al., 2004). MDGA expression is both spatially and temporally regulated. For example, high MDGA1 expression is detected in the cortex and hindbrain as early as rat embryonic day 15 (E15), expression is highest in the basilar pons, hippocampus, amygdala, olfactory bulb and superficial layers of the cortex at postnatal day 1 (P1), and by P7 expression is similar to P1 but with relative increases in the cerebellum and superior and inferior colliculi (Litwack et al., 2004). MDGA2 appears to have broader but lower expression, being present at high levels in the basilar pons and low levels throughout most of the brain including all MDGA1-expressing areas (Lein et al., 2007; Litwack et al., 2004). In adult brain, MDGA1 exhibits much higher expression than MDGA2 (Lein et al., 2007). MDGA1 and 2 also show restricted and non-overlapping expression in the spinal cord. Further biochemical characterization showed that MDGA1 is indeed linked to the plasma membrane by GPI anchors, is subject to *N*-glycosylation and is localized to lipid rafts

when transfected into non-neuronal cells (Diaz-Lopez et al., 2005; Litwack et al., 2004).

MDGAs do not bind in a homophilic manner, and a heterophilic binding partner has not been discovered (Fujimura et al., 2006). A soluble version of MDGA1 (lacking the GPI-linkage) preferentially binds to axon-rich areas in both the peripheral and central nervous systems in embryonic chick. Domain analysis showed that the MAM domain was required for binding to these axon-rich areas, while the first four Ig domains were required for binding to differentiating muscle tissue. As MDGA1 is not expressed in muscle, this binding was presumably heterophilic, although the binding partner was not identified (Fujimura et al., 2006).

1.6.2 MDGAs in Cortical Migration and Organization

Further *in situ* hybridizations showed that MDGA1 is expressed in layer 2/3 cortical neurons early in development, during their radial migration and settling in the cortical plate (Takeuchi and O'Leary, 2006). To investigate a possible role of MDGA1 in the migration of these cortical neurons, small interference RNA (RNAi) was delivered to mice at E15.5 using *in utero* electroporation, which was expected to target mostly layer 2/3 neurons. The overall cortical architecture and morphology appeared normal at P0 for both control and RNAi transfected neurons. However, while control transfected neurons showed a normal distribution, RNAi transfected cells were still deep in the cortical plate or in the intermediate zone, suggesting that reducing MDGA1 expression inverts the distribution of layer 2/3 neurons from superficial to deep (Takeuchi and O'Leary, 2006). It appears that this is a cell-autonomous effect, as co-expression of rat MDGA1 partially rescued the migration

defects in layer 2/3 neurons, and RNAi transfection of deep layer neurons that do not express MDGA1 had no effect on their migration (Takeuchi and O'Leary, 2006). Further analysis of MDGA1 expression patterns during development showed that at P7, although it is expressed in layer 2/3 neurons throughout the cortex, it is also expressed in layer 4 and a few deep layer 6a neurons specifically in the primary somatosensory area S1 (Takeuchi et al., 2007a). This differential expression of MDGA1 in layer 4 S1 neurons, which can be detected as early as P0, suggests it may play a role in barrel patterning. Transient MDGA1 expression was also detected in Cajal-Retzius neurons earlier in development (E9.5-E13.5), and also in some of the earliest diencephalic and mesencephalic neurons; these early expression patterns suggest that MDGA1 may have important functions in various aspects of forebrain development, such as migration and laminar or area patterning (Takeuchi et al., 2007a).

A role for MDGA1 in radial migration and positioning was further confirmed in a study using an MDGA1 mutant mouse. Part of the N-terminal domain (including the signal sequence) of MDGA1 was replaced by the LacZ reporter cassette, resulting in abolished expression of MDGA1 in homozygous mutant mice (Ishikawa et al., 2011). Homozygous mice were fertile and reached adulthood without any obvious abnormalities, and gross brain morphology appeared normal at P14, with MDGA1-negative mutant cells (expressing LacZ) having similar positions as wild type littermates. However, when brain slices were examined earlier, during corticogenesis, MDGA1-negative cells were retained in deeper layers of the cortical plate compared to wild type neurons. Altered localization was still present by P0, but

MDGA-1 mutant neurons had attained correct positioning in the upper layers of the cortex by P7, suggesting that MDGA1 deficiency delayed migration (Ishikawa et al., 2011). However, it was not possible to tell whether the LacZ-expressing neurons at P7 were the same ones that had delayed migration, or whether these were cells that had already attained the correct position and subsequently began expressing LacZ under the MDGA1 promoter. There are a few discrepancies between this study and the siRNA study described above: essentially all siRNA-transfected neurons did not migrate properly and completely failed to attain a proper position by P0, whereas only a subset of cortical neurons in MDGA1 mutant mice had migrational deficits, which were already partially improved at P0 in the mutant mice (Ishikawa et al., 2011; Takeuchi et al., 2007a). Nevertheless, it is clear that MDGA1 does play a role in migration of at least some cortical neurons. Further avenues of inquiry in this area could focus on the mechanism by which MDGA1 influences migration in a cell-autonomous manner, as well as possible roles for MDGA2.

1.6.3 MDGAs in Neurodevelopmental Disorders

Interestingly, the MDGA family has recently been linked to neurodevelopmental disorders. An initial study linked six different SNPs in MDGA1 to schizophrenia; these SNPs were located in 3'-untranslated regions or in introns (Kahler et al., 2008). A subsequent independent study found one of these same SNPs and ten new MDGA1 SNPs in association with schizophrenia and bipolar disorder, most of which were also in 3'-untranslated regions or in introns (Li et al., 2011). Lastly, another study linked exonic deletions in MDGA2 to autism, and also found deletions in other genes previously linked to ASDs, including neurexin-1

(Bucan et al., 2009). The various MDGA2 deletions are predicted to result in truncation or complete deletion of MDGA2, and, like many other ASD-associated mutations, show imperfect segregation with disease (Bucan et al., 2009). These new results, together with the growing body of evidence linking synaptic protein mutations to neurodevelopmental disorders, suggests that the MDGA family may also have roles at synapses later in development.

1.7 Thesis Hypothesis and Objectives

The formation and development of synapses involves a number of molecular cues. Cell adhesion molecules may play a particularly important role by inducing the initial formation of pre- and postsynaptic specializations, and by participating in synapse maturation and stability processes. Despite the large number of synaptic cell adhesion molecules that have been reported, knockout mice studies suggest that there are still undiscovered synapse-modifying proteins. The overall goal of this thesis was to discover and characterize new molecules that can influence synapse development.

Using an un-biased screen for synaptogenic proteins, calsyntenin-3 was discovered, while MDGAs were postulated to play roles in synaptic development based on protein structure, expression patterns and links to neurodevelopmental disease. This led to the hypothesis that both calsyntenins and MDGAs were cell-surface proteins that could potentially impact synapse development. Thus, the objective of this work was to study the effects of calsyntenins and MDGAs in developing cultured hippocampal neurons, and to determine the mechanisms by which these effects are mediated.

Chapter 2: Calsyntenins

2.1 Introduction

Synapses are the basic units of communication in the brain, and their development requires the coordinated efforts of a number of different molecular players. These molecules can be secreted or cell-associated, and orchestrate a series of steps including axon targeting, priming of the pre- and postsynaptic partners, stabilizing initial contact and inducing differentiation of pre- and postsynaptic compartments (Waites et al., 2005). Recently, many cell adhesion molecules (CAMs) have been shown to be induction factors in synaptogenesis. The best-characterized, postsynaptic neuroligins and their presynaptic binding partners neurexins, are able to induce pre- and postsynaptic specializations, respectively (Graf et al., 2004; Scheiffele et al., 2000). These initial studies suggested that a single molecule presented to axons or dendrites could stimulate many aspects of pre- or postsynaptic assembly. However, both neuroligin-1,-2,-3 triple knockout and α-neurexin-1,-2,-3 triple knockout mice still form synapses, despite major defects in neurotransmission (Missler et al., 2003; Varoqueaux et al., 2006), suggesting that there must be other molecular players that participate in synaptogenesis.

The neuron-fibroblast co-culture assay, in which a single protein is expressed on the surface of fibroblasts and presented to developing axons or dendrites, has been a valuable tool to discover new inductive synaptic CAMs. Such co-culture assays initially identified synaptogenic activity for neurexins and neuroligins (Graf et al., 2004; Scheiffele et al., 2000), SynCAMs (Biederer et al., 2002), EphBs and ephrins (Aoto et al., 2007; Kayser et al., 2006), and netrins G ligand (Kim et al.,

2006), as well as others. New evidence from mouse models, including the phenotypes from neuroligin and neurexin knockout mice, suggest that the co-culture screen may in fact be quite permissive and allow for the identification of molecules involved in synaptic maturation in addition to identifying those required for initial formation *in vivo*.

In this study, the fibroblast-neuron co-culture assay was used as an unbiased screening method to search for new synaptogenic proteins. A set of full-length, size-selected cDNA expression libraries were created from developing rat brain, which were then transfected into fibroblasts and co-cultured with developing hippocampal neurons. This led to the identification of a number of novel protein families able to induce presynaptic differentiation. The Craig lab previously reported the characterization of one of these protein families, the LRRTMs (Linhoff et al., 2009). Here, the characterization of calsyntenin-3, another positive protein from this screen, is presented.

Calsyntenins are a family of three proteins conserved in vertebrates; orthologs also exist in *C. elegans* and *Drosophila* (Hintsch et al., 2002; Vogt et al., 2001). Calsyntenin-1 is expressed highly in the brain and is also found in other tissues, while calsyntenin-2 and -3 are expressed exclusively in the brain at moderate and high amounts, respectively (Hintsch et al., 2002; Vogt et al., 2001). Within the brain, the calsyntenins also show differential patterns of expression, and all three are expressed in the hippocampus (Hintsch et al., 2002). At the synaptic level, calsyntenins appear to be present at the postsynaptic compartment, mostly at glutamatergic but also at some GABAergic synapses (Hintsch et al., 2002).

Calsyntenins are type I transmembrane proteins and contain two extracellular cadherin domains and one LNS domain (Hintsch et al., 2002). Interestingly, calsyntenin-1 was initially discovered as a protein secreted in developing chick spinal cord neurons (Vogt et al., 2001). Further characterization showed that calsyntenins are proteolytically processed in a similar manner to amyloid precursor protein (APP), being cleaved in the extracellular domain by ADAM10 or ADAM17, followed by intramembrane cleavage by γ-secretase (Araki et al., 2004; Araki et al., 2003; Hata et al., 2009). In fact, calsyntenin-1 forms a tripartite complex with Mint2/X11L and APP, and formation of this complex reduces the processing of both calsyntenin-1 and APP (Araki et al., 2004; Araki et al., 2003). It has been suggested that the coordinated processing of APP and calsyntenins could be a useful diagnostic tool for Alzheimer's disease, as cleavage products from calsyntenins can be detected in cerebrospinal fluid (Hata et al., 2009; Konno et al., 2011). Calsyntenin-1 also binds to kinesin light chain 1 and thus acts as a cargo docking protein for anterograde transport (Konecna et al., 2006). In this capacity, calsyntenin-1 plays a role in APP trafficking as well (Araki et al., 2007; Ludwig et al., 2009; Steuble et al., 2010). Calsyntenin-2, on the other hand, has been linked to episodic memory in humans (Jacobsen et al., 2009; Papassotiropoulos et al., 2006; Preuschhof et al., 2010), while the *C. elegans* ortholog CASY-1 is necessary for a number of learning paradigms in worms (Hoerndli et al., 2009; Ikeda et al., 2008).

To date, very little has been published regarding a physiological role for calsyntenin-3. One study in humans showed a down-regulation of calsyntenin-3 in human familial Alzheimer's disease patients (Ringman et al., 2012), while another

showed up-regulation of calsyntenin-3 in response to treatment with Aβ peptides and in the brain of a mouse model for Alzheimer's disease (Uchida et al., 2011). Thus, it is unclear at this time if calsyntenin-3 is at all physiologically related to APP, despite undergoing similar processing steps.

In this study, calsyntenin-3, but not calsyntenin-1 or -2, is shown to induce both excitatory and inhibitory presynaptic specializations in axons when presented to developing neurons in co-culture. This synaptogenic activity is mediated through the membrane-anchored extracellular domain of calsyntenin-3 and does not require the intracellular domain. Furthermore, overexpression of calsyntenin-3 in neurons drastically increases the clustering of both excitatory and inhibitory presynaptic markers. Interestingly, calsyntenin-3 binds α-neurexin with high affinity, suggesting that calsyntenin may mediate trans-synaptic signaling through neurexins. This finding opens up new avenues of study for calsyntenin-3, and places it in league with the growing number of postsynaptic neurexin binding partners involved in synaptic development, including neuroligins, LRRTMs and GluRδ2-Cbln1 (Craig and Kang, 2007; de Wit et al., 2009; Dean et al., 2003; Ko et al., 2009; Matsuda et al., 2010; Siddiqui et al., 2010; Uemura et al., 2010).

2.2 Experimental Procedures

2.2.1 Un-biased Co-Culture Screen

The creation of full-length, size-selected expression libraries, the generation of plasmid pools for screening, PCR analysis of synaptogenic cDNA pools and breaking down of positive pools has been described in detail elsewhere (Linhoff,

2008; Linhoff et al., 2009). The current study began once calsyntenin-3 had been identified and isolated as a positive clone able to induce presynaptic clustering in contacting axons.

2.2.2 DNA Constructs

Calsyntenin-3 cDNA was isolated from the positive clone in the screen, and subcloned into the pcDNA3 vector under the cytomegalovirus (CMV) promoter. In order to measure surface expression levels of constructs and to track the N-terminal cleavage product, the sequence for the Myc-tag (amino acids (aa) EQKLISEEDL) was inserted after the 20 amino acid signal sequence in full length calsyntenin-3 (957 aa) to make Myc-calsyntenin-3 (Myc-C3), using the QuikChange site-directed mutagenesis kit (Stratagene). In order to directly detect expression in cells, the cyan fluorescent protein sequence (CFP) (252 aa) was directly fused in-frame to the C-terminus of both calsyntenin-3 and Myc-C3 to produce C3-CFP and Myc-C3-CFP, respectively, by subcloning into the pECFP-N1 vector (Clontech). The Myc-C3-CFP vector formed the base for the subcloning of a series of deletion constructs, which were produced by a PCR amplification of desired insert or overlap PCR methods to produce the insert, followed by restriction digest and ligation into the pECFP-N1 or pcDNA3 vectors; all these constructs were under the control of the CMV promoter. These constructs were: Myc-C3secreted, (Δ aa 851-957), in which the full length sequence was truncated just before transmembrane (TM), thus deleting the TM, intracellular domain (ICD) and CFP; C3-intracellular-CFP, (Δ aa 1-869), in which the entire extracellular and TM domains were removed; Myc-C3EXTM-CFP, (Δ aa 870-957), in which only the ICD was deleted, and the C-terminus of TM domain was re-

fused in-frame to the CFP domain; Myc-C3ΔPCS-CFP, (Δ aa 805-824), PCS for "primary cleavage site," in which the 20 amino acids flanking the reported/putative cleavage site were deleted using site-directed mutagenesis; Myc-C3EX-CD8-CFP, (Δ aa 815-957), in which the majority of the extracellular domain (ending ~30 amino acids upstream of the putative cleavage site, at the –PSHV sequence) was fused to only the TM domain of the CD8 protein via a short linker sequence of three glycines, which was fused to intracellular CFP; Myc-C3EX-CD8-C3IN, (Δ aa 815-869), was subcloned from Myc-C3EX-CD8-CFP by replacing the CFP sequence with the calsyntenin-3 ICD sequence, thus resulting in a construct only lacking a short juxtamembrane extracellular sequence and the calsyntenin-3 TM domain; MycC3CADonly-CFP, (Δ aa 255-957), in which the extracellular domain until the end of the second cadherin domain was fused to the CD8 TM domain via a short linker sequence of three glycines, which was fused to intracellular CFP; MycC3partΔCAD-CFP, (Δ aa 63-128), in which approximately the first 2/3 of the first cadherin domain was deleted; Myc-C3ΔCAD-CFP, (Δ aa 51-244), in which the N-terminal domain from after the signal sequence-Myc and first extracellular region until the end of the second cadherin domain was deleted; Myc-C3ΔLNS-CFP, (Δ aa 335-542), in which the entire LNS-like domain was deleted; Myc-C3LNSDN/AA-CFP, in which the aspartic acid (D) and asparagine (N) residues at positions 475 and 476 in the LNS domain were mutated to two alanine (A) residues by site-directed mutagenesis; and Myc-C3LNSQ/K-CFP, in which the glutamine (Q) residue at position 441 in the LNS domain was mutated to a lysine (K) residue by site-directed mutagenesis. Domains (signal sequence, CAD, TM, etc.) were delineated according to the published

sequences (Hintsch et al., 2002) or by homology searches. Please see Figure 2.5 for a diagram of these deletion constructs.

Calsyntenin-1 and calsyntenin-2 full length mouse cDNA sequences were obtained from Open Biosystems and subcloned into the pcDNA3 vector. Myc-C1, Myc-C2, C1-CFP, C2-CFP, Myc-C1-CFP and Myc-C2-CFP were generated in parallel methods to those used for calsyntenin-3. In addition, extracellular only versions were created in a similar method to Myc-C3EX-CD8-CFP, by fusing the extracellular domain (just upstream of the putative cleavage sites) from Myc-C1-CFP and Myc-C2-CFP to the CD8 TM domain via a short linker sequence of three glycines, which was fused to intracellular CFP to produce Myc-C1EX-CD8-CFP, (Δ aa 826-979), and Myc-C2EX-CD8-CFP, (Δ aa 810-966), respectively. The plasmid encoding Clstn3-Fc was subcloned from C3-CFP into the pc4-sp-Fc1 pcDNA4 vector, allowing for fusion of the extracellular domain of calsyntenin-3 (aa 21-847) to the human IgG Fc portion, followed by a stop codon.

Previously described plasmids used from our lab include CFP-neuroligin2 (or CFP-Nlg2), N-cadherin-CFP, HA-neurexin1α, HA-CD8, mCFP (membrane CFP) and solCFP (soluble CFP) (Gauthier et al., 2011; Graf et al., 2004; Kang et al., 2008; Linhoff et al., 2009).

2.2.3 Cell Culture and Transfection

Dissociated primary hippocampal neuron cultures were prepared from embryonic day 18 rat embryos as described previously (Banker and Goslin, 1998; Kaech and Banker, 2006). Neurons were plated at a density of 300,000 cells per dish on poly-L-lysine coated coverslips and inverted over a feeder layer of glia in

60mm culture dishes. To prevent overgrowth of glia, cytosine arabinoside (5 μM) was added to neuron cultures at 2 d. Serum-free media was also supplemented with 100 μM APV (Research Biochemicals) to prevent excitotoxicity. For overexpression studies, neurons were transfected with 5-8 μg of DNA per dish at day *in vitro* (DIV) 8-9 using the ProFection Mammalian Transfection System (Promega). Neurons were fixed at 14 DIV.

COS7 and HEK293T cells were cultured in DMEM-H media with 10% fetal bovine serum. All transfections of COS7 cells for binding assays, surface expression assays and co-cultures were performed using TransIT-LT1 Transfection Reagent (Mirus), using 1-2 μg of DNA. Co-cultures of primary hippocampal neurons with COS7 cells were performed as described previously (Graf et al., 2004). Briefly, transfected COS7 cells were trypsinized on the day after transfection and resuspended in conditioned neuron culture media. Neurons grown for 9-10 DIV were inverted in their home dish and COS cells were seeded into the neuron dish. After 2 h, the neuron coverslips were flipped back over so the neurons and COS cells were facing the glial feeder layer. After 20-24 h of co-culture, cells were fixed for analysis.

2.2.4 Production of Soluble Clstn3-Fc fusion Protein

Expression of Clstn3-Fc protein was performed by transfecting HEK293T cells with the encoding plasmid, and culturing in DMEM with 10% FBS and 0.5 mg/ml Zeocin (Invitrogen). After 21-day selection with Zeocin, medium was replaced with serum-free AIM V synthetic medium (Invitrogen). The conditioned medium was collected every 2-3 days for three weeks and frozen at -80°C, for a total of 300-400 mL. Fc fusion protein was purified using protein-G sepharose 4 fastflow columns

(GE Healthcare) and concentrated in PBS with Centricon filters (Millipore). Purified Fc fusion proteins were immunoblotted, visualized by chemiluminescence using a Bio-Rad gel documentation system, and quantified by densitometry relative to a human IgG standard curve.

2.2.5 Western Blotting

For analysis of cleavage of various calsyntenin-3 constructs, COS7 cells were transfected as described above. After 24-48 hours of expression, the media was collected, treated with protease inhibitor cocktail tablets and placed on ice (Roche), and the cells were washed with phosphate buffered saline then scraped into lysis buffer (1% triton X-100, 150mM NaCl, 20mM Tris pH 7.4, 1mM DTT, 1mM EDTA, plus protease inhibitor tablet) (Sigma, Roche). Lysates were centrifuged at 16000 x g for 15 min at 4°C and the supernatant was collected. The protein concentrations of both media and lysate fractions were determined using the Bio-Rad Protein Assay kit (Bio-Rad), using BSA as a standard (Sigma). Protein concentrations were normalized between samples and loaded into 10% polyacrylamide gels with an equal volume of loading buffer, using Magic Mark XP protein ladder as a marker (Invitrogen). Gels were run and transferred using the Hoeffer Mini Western Electrophoresis System with Transfer Tank (Hoeffer). Transfer was completed onto Immobilon P membranes (Millipore), blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 (Sigma) and incubated with primary (anti-Myc mouse IgG1; Invitrogen), and secondary (Goat anti-mouse HRP conjugate; Millipore) antibodies. Immunoblots were detected using the SuperSignal Chemiluminescent kit (Thermo

Scientific) and visualized by chemiluminescence using a Bio-Rad gel documentation system.

2.2.6 Immunocytochemistry

For staining COS cells, neuron-COS co-cultures or neuron cultures, the following protocol was used. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4) for 15 min followed by permeabilization with PBST (PBS + 0.25 % Triton X-100) or in -20°C methanol for 10 min. They were then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C, followed by incubation with primary antibodies in blocking solution (overnight, 20°C) and secondary antibodies (45 min, 37°C). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

For the synaptotagmin I antibody uptake assay, neurons were incubated live with antibodies to the synaptotagmin luminal domain (1:200; IgG1; clone 604.2; Synaptic Systems) for 30 min in culture media at 37°C in a 5% CO2 incubator.

For surface labeling of HA- or Myc- signals in neurons, the same protocol was followed except that cells were incubated with anti-HA (1:500; IgG2b; clone 12CA5; Roche) or anti-Myc (1:500; mIgG1; Invitrogen) antibodies for 1 h at 37°C following fixation in parafix solution, but prior to permeabilization with PBST.

For determining surface expression in COS7 cells, anti-Myc antibody (1:500; mIgG1; Invitrogen) in “binding buffer” (see below) was incubated with cells for 30 min at 20°C. Cells were then fixed in parafix solution, permeabilized, blocked and incubated with primary and secondary antibodies as described above.

The following polyclonal primary antibodies were used: rabbit anti-synapsin I (1:2000; Millipore; AB1543P), rabbit anti-VGlut1 (1:2000; Synaptic Systems; 135 302), rabbit anti-VGAT (1:1000; Synaptic Systems; 131 003). The following mouse monoclonal antibodies were used: anti-PSD-95 family (1:500; IgG2a; clone 6G6-1C9; Thermo Scientific; recognizes PSD-95, PSD-93, SAP102 and SAP97), anti-gephyrin (1:500; IgG1; mAb7a; Synaptic Systems), anti-HA (1:1000; IgG2b; clone 12CA5; Roche), anti-Myc (1:1000, IgG1; Invitrogen), anti-bassoon (1:1000; IgG2a; Stressgen; VAM-PS003) and anti-synaptophysin (1:1000; IgG1; BD Biosciences; 611880). For labeling dendrites, anti-MAP2 (1:4000, chicken polyclonal IgY; Abcam; ab5392) was used. For labeling axons, anti-tau-1 (1:2000; mIgG2a; clone PC1C6; Millipore; MAB3420; recognizes dephosphorylated tau) was used.

Secondary antibodies were raised in goat against the appropriate species and monoclonal isotype, highly cross-absorbed and conjugated to Alexa-488, Alexa-568 and Alexa-647 dyes (1:500, Invitrogen). AMCA (7-amino-4methylcoumarin-3-acetic acid)-conjugated anti-chicken IgY (donkey IgG; 1:200; Jackson ImmunoResearch; 703-155-155) was used for visualizing dendrites.

2.2.7 Binding Assays

To assess binding between HA-neurexin-1α and calsyntenin-3, COS7 cells growing on coverslips were transfected with HA-neurexin-1α and allowed to express for 24 h. Cells were incubated with fusion protein Clstn3-Fc live for 1 h at 20°C, followed by anti-HA antibodies (1:500; IgG2b; clone 12CA5; Roche) for 30 min. Binding was assayed in the following “binding buffer”: 168 mM NaCl, 2.6 mM KCl, 10 mM HEPES, pH 7.2, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, and 100 µg/ml

BSA. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4)) for 15 min at 20°C then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C. This was followed by incubation with secondary antibodies FITC-conjugated donkey anti-human IgG (H+L) (1:100, Jackson ImmunoResearch) and Alexa-568 anti-IgG2b (1:1000, Invitrogen) for 1 h at 37°C to visualize bound-Fc protein and surface HA, respectively. Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

2.2.8 Imaging, Image Analysis and Statistical Analysis

Images were acquired on a Zeiss Axioplan2 microscope with a 40X 1.30 NA oil objective, a 63X 1.4 NA oil objective or a 25X 0.8 NA oil objective and Photometrics Sensys cooled CCD camera using Metamorph imaging software (Molecular Devices) and customized filter sets. Images were initially acquired as 12 bit grayscale and were prepared for presentation using Adobe Photoshop (Adobe Systems). For quantification, sets of cells were fixed and stained simultaneously and imaged with identical settings. All image acquisition, analysis and quantification were done blind to the experimental condition.

For quantifying most co-culture experiments, a visual method was used. Transfected COS cells were selected for measuring based on moderate expression (based on CFP expression or co-expression), normal morphology and contact with neurites (as viewed under phase contrast). A selected cell was then viewed under fluorescence to determine the presence of presynaptic protein clustering (synapsin, VGlut1, VGAT puncta), in the absence of either dendrites (by MAP2 staining) or

postsynaptic clusters (by PSD95 and/or gephyrin puncta). If presynaptic clusters were present over the transfected cell without MAP2 or apposed postsynaptic clusters, the cell was scored as positive, whereas if presynaptic clusters were apposed to a MAP2 positive neurite or postsynaptic clusters, the cell was scored as negative. Many hundreds of cells were scored across independent experiments.

For quantitation of tau and synapsin signals in co-culture assays, regions were created around the expressing COS cells that excluded MAP2-positive areas, and the total intensity and area of all puncta in the synapsin channel and all crossing axons in the tau channel were each thresholded and measured. The COS area measurements, created from the delineated COS cell region, were used to normalize measures to COS7 cell area. Measures were corrected for off-cell background.

To determine the binding affinity of Clstn3-Fc to surface-expressed HA-neurexin-1α, regions were drawn around the perimeter of each COS cell, and the average intensity values of bound protein and expressed protein were measured within the region. Average off-cell background measures were subtracted from these values to yield corrected average intensity values for bound protein and expressed protein. Similar methods were employed to determine surface (Myc) expression compared to total expression of CFP-tagged proteins in COS cells.

For analysis of neurons in the overexpression experiments, neurons were chosen for imaging based on Myc / HA or CFP signal, as well as healthy morphology under phase contrast and MAP2 channels. Neighbouring cells for overexpression analysis were chosen based on similar MAP2 staining. During analysis, regions

were created around single expressing or non-expressing dendrites and thresholded in the synapsin, VGAT or VGlut1, and gephyrin or PSD95 channels. Total number of puncta and area were measured for each channel. Average intensity for surface Myc signal was also measured in the selected region. Measures were corrected for off-cell background and normalized to dendrite length.

Analysis was performed using Metamorph (Molecular Devices), Excel (Microsoft) and GraphPad Prism (GraphPad Software). Statistical comparisons were made with Student's unpaired t-test or one-way ANOVA with post hoc Bonferroni's multiple comparison test, as indicated in figure legends. All data are reported as the mean ± standard error of the mean (SEM).

2.3 Results

2.3.1 An Expression Screen for Synaptogenic Molecules Isolated Calsyntenin-3

To search for new synaptogenic proteins, our lab developed a screen using the fibroblast – neuron co-culture assay, which has been described elsewhere (Linhoff, 2008; Linhoff et al., 2009). Briefly, mRNA from rat forebrain was isolated at the peak of synaptogenesis, P11, and was used to generate full-length cDNA using the biotinylated cap-trapper method (Carninci et al., 1996; Micheva and Beaulieu, 1996). The expression libraries were generated from different size fractions of cDNA. Pools were then transfected into COS cells and cultured with hippocampal neurons. Co-cultures were immunostained for the presynaptic protein synapsin1 to detect synaptic vesicle clustering in axons contacting transfected COS cells. In order

to confirm that *bona fide* neuron-neuron synapses were not counted as false positives, co-cultures were co-immunostained for the postsynaptic markers PSD-95 family and gephyrin. Figure 2.1 (a) outlines the experimental design for the co-culture screen. A positive clone in the screen showing synapsin clustering in the absence of postsynaptic clustering was identified visually; calsyntenin-3 was initially discovered this way in pool PC151 from the 3-4 kb library (Figure 2.1, b). PCR screening of this pool for cDNAs of known synaptic proteins, including neuroligins and LRRTMs, showed that neither were present, suggesting the presence of a novel synaptogenic protein (Figure 2.1, d, e). When PC151 was broken down until a single clone was isolated, co-cultures showed increased presynaptic clustering (Figure 2.1, c). Sequencing of this clone identified it as calsyntenin-3, which has previously been reported as a member of a family of type 1 transmembrane proteins that are expressed in the brain and exhibit postsynaptic localization (Hintsch et al., 2002; Vogt et al., 2001). All three calsyntenin family members have a large extracellular domain containing two cadherin repeats and an LNS-like domain, followed by a transmembrane region and a short cytoplasmic domain. Interestingly, calsyntenins can be cleaved in the extracellular domain by ADAM proteases, followed by intramembrane cleavage by γ-secretase, raising intriguing possibilities for the regulation and mechanism by which they may induce presynaptic differentiation (Araki et al., 2004; Hata et al., 2009; Vogt et al., 2001).

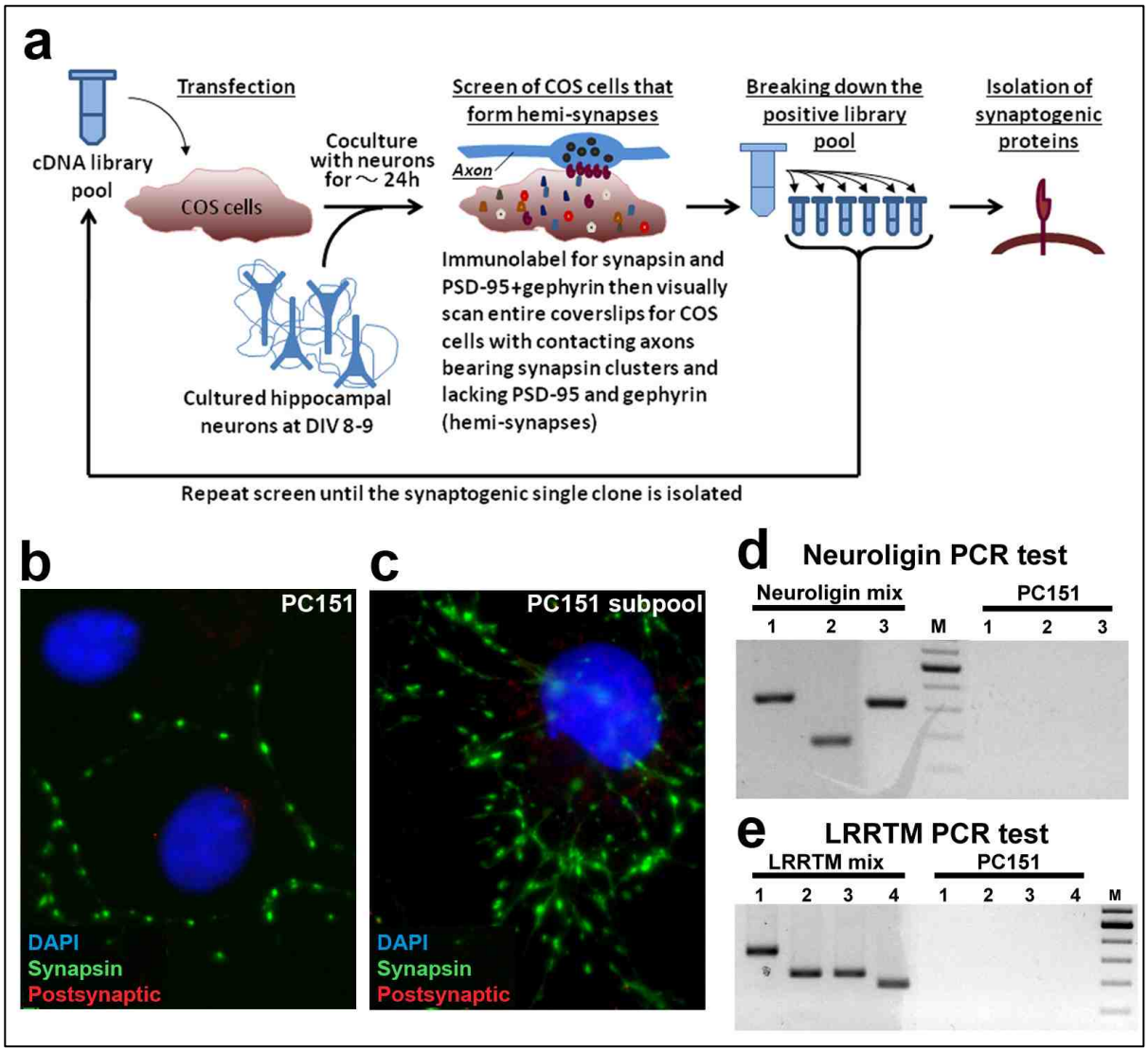

Figure 2.1: An Unbiased Screen Identified Calsyntenin-3 as a Synaptogenic Factor
(a) Flow diagram illustrating the experimental protocol for expression screen leading to discovery of calsyntenin-3. Expression pools were transfected into COS cells in 12-well tissue culture plates, and transfected COS cells were then co-cultured with hippocampal neurons for ~24 h. Co-culture coverslips were then fixed and immunolabeled for synapsin, PSD95 and gephyrin. Coverslips were visually scanned on a fluorescent microscope for the presence of COS cells inducing presynaptic synapsin clustering without apposed postsynaptic PSD95 and gephyrin. (b) Merged image of the PC151 co-culture showing clustering of presynaptic synapsin (green) over a COS cell (nucleus stained blue with DAPI), without apposing postsynaptic markers (in red). (c) Merged image of PC151 pool which was broken down to smaller subsets. Enrichment of the synaptogenic clone is evident by the increase in synapsin clustering. (d) PC151 cDNA pool was tested for the presence of neuroligins by PCR. Primer sets 1, 2 and 3 indicate the neuroligin isoform the primers were designed to detect. A mix of neuroligin cDNAs served as the positive control. No signal was evident in PC151 pool. (e) PC151 cDNA pool was tested for the presence of LRRTMs by PCR. Primer sets 1, 2, 3 and 4 indicate the LRRTM isoform the primers were designed to detect. A mix of LRRTM cDNAs served as the positive control. No signal was evident in PC151 pool. Panel (a) was reproduced with permission from Takahashi et al., Neuron, 2011, and panels (b-e) were reproduced with permission from the doctoral dissertation of Michael Linhoff, Washington University in St. Louis, 2008.

2.3.2 Quantitation of the Synaptogenic Activity of Calsyntenins

In order to be able to track both the N- and C-terminal portions of calsyntenins, the Myc tag was inserted after the signal sequence at the N-terminus and CFP was fused to the C-terminus of calsyntenin-3. Expression of cloned rat Myc-calsyntenin-3-CFP (Myc-C3-CFP) in COS cells in co-culture with hippocampal neurons showed that the contacting axon terminals clustering synapsin in the absence of dendrites (identified by MAP2 staining) also uptake an antibody against the luminal domain of synaptotagmin, suggesting that these terminals undergo synaptic vesicle exocytosis (Figure 2.2, a). As the other two calsyntenins are quite similar to calsyntenin-3 (sharing ~ 50% similarity in mouse) (Hintsch et al., 2002), it was hypothesized that they also might induce presynaptic clustering. Therefore, calsyntenin-1 and calsyntenin-2, with and without N-terminal Myc and C-terminal CFP tags were also cloned and tested in co-culture. Interestingly, neither calsyntenin-1 nor calsyntenin-2 shares the synaptogenic activity of calsyntenin-3 in hippocampal co-cultures, showing no evidence of synapsin clustering over transfected cells, similar to the negative controls N-cadherin-CFP (Figure 2.2, b and c). Compared to the well-established synaptogenic activity of neuroligin-2 (used as a positive control), calsyntenin-3 has somewhat lower activity in co-culture, and neither the Myc nor the CFP tags has any significant effect on this activity when measured using the visual scoring method (see Experimental Procedures for details) (Figure 2.2, c). The synaptogenic activity of calsyntenin-3 is not just specific for synapsin clustering, as other presynaptic proteins such as synaptophysin and bassoon can also be clustered by Myc-C3-CFP expressing COS cells (Figure 2.2, d).

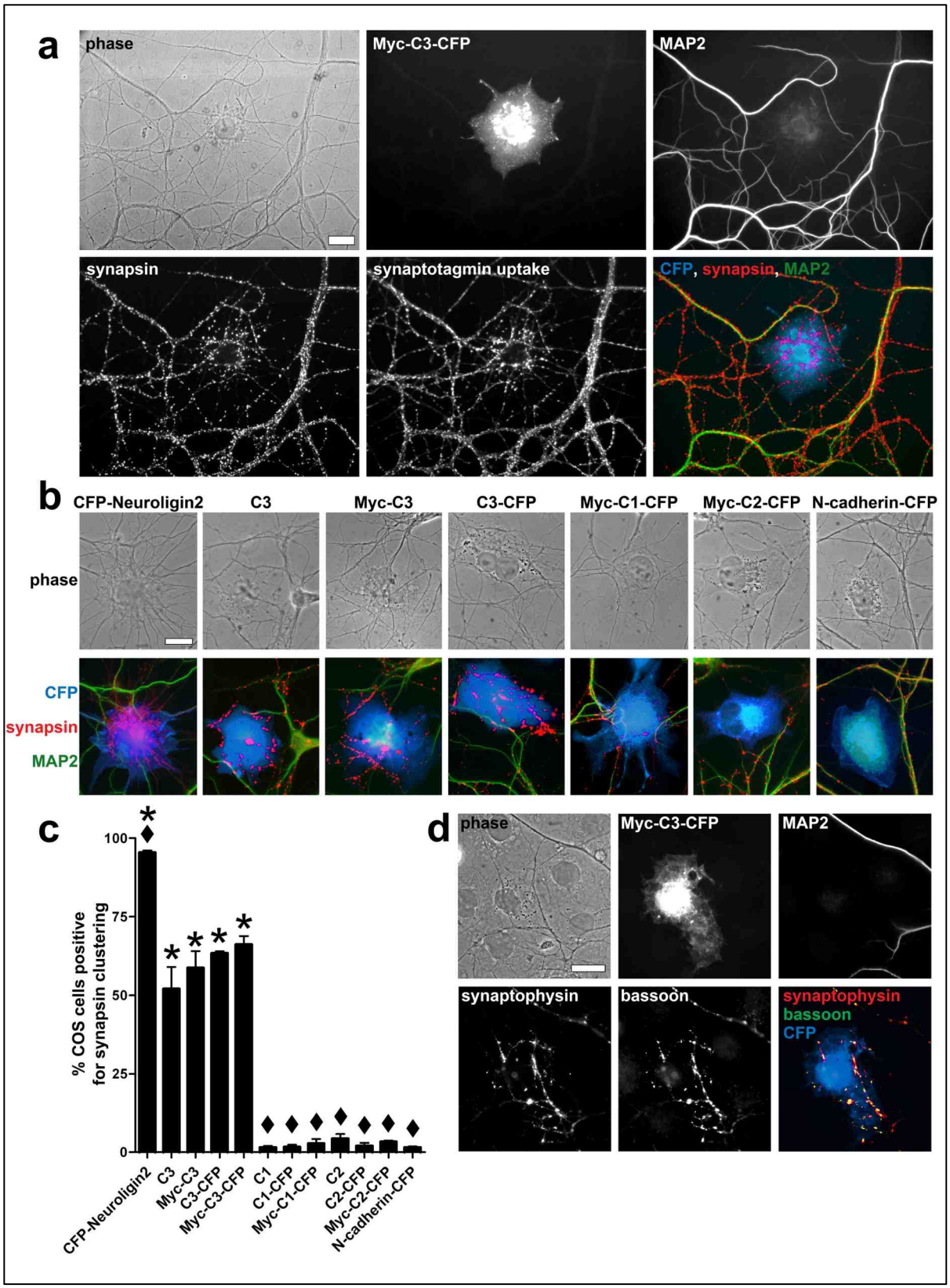
a
phase
Myc-C3-CFP
MAP2
synapsin
synaptotagmin uptake
CFP, synapsin, MAP2
b
CFP-Neuroligin2
C3
Myc-C3
C3-CFP
Myc-C1-CFP
Myc-C2-CFP
N-cadherin-CFP
phase
CFP
synapsin
MAP2
c
% COS cells positive for synapsin clustering
100
75
50
25
0
CFP-Neuroligin2
C3
Myc-C3
C3-CFP
Myc-C3-CFP
C1
C1-CFP
Myc-C1-CFP
C2
C2-CFP
Myc-C2-CFP
N-cadherin-CFP
d
phase
Myc-C3-CFP
MAP2
synaptophysin
bassoon
synaptophysin
bassoon
CFP

Figure 2.2: Calsyntenin-3, but not Calsyntenin-1 and -2, Induces Presynaptic Clustering in Hippocampal Co-cultures
(a) Calsyntenin-3 expressed in COS cells and tagged with N-terminal Myc and C-terminal CFP tags (Myc-C3-CFP) (top middle panel) can induce the formation of presynaptic terminals (shown here by synapsin clustering in bottom left panel) in contacting axons. These terminals lack apposing dendrites (shown by MAP2 staining in top right panel). The terminals are also positive for synaptotagmin antibody uptake (bottom middle panel), suggesting that they are active terminals. Bottom right panel shows combined image of Myc-C3-CFP transfected COS cell (CFP in blue), MAP2-positive dendrites (green) and synapsin (red). (b) Co-cultures show that, like neuroligin-2 (first column), calsyntenin-3 expressed in COS cells can cluster synapsin in hippocampal co-cultures (second column). Neither the Myc nor CFP tags interfere with this activity (third and fourth column). However, calsyntenin-1 and calsyntenin-2 do not cluster presynaptic proteins in the co-culture assay (fifth and sixth column), like the negative control N-cadherin-CFP (last column). All bottom row images show combined images of synapsin (red), tagged protein or co-transfected CFP (blue) and dendrites by MAP2 (green). (c) Quantitation of presynaptic induction of calsyntenin family members, by measuring % of transfected COS cells clustering synapsin without MAP2. ANOVA, $P < 0.0001$, $n \geq 3$ experiments counting ≥ 100 cells per experiment; *$P < 0.001$ compared to negative control N-cadherin-CFP by post-hoc Bonferroni test.♦ $P < 0.001$ compared to Myc-C3-CFP by post-hoc Bonferroni test. Post-hoc Bonferroni test also showed no significant difference between C3 and any of the tagged C3 constructs (Myc-C3, C3-CFP and Myc-C3-CFP). Results are expressed as mean ± SEM. (d) Myc-C3-CFP in COS cells can also cluster other presynaptic proteins including synaptophysin (bottom left panel) and bassoon (bottom middle panel). Bottom right panel shows combined image with synaptophysin (red), bassoon (green) and transfected COS (CFP in blue). Scale bars are 20 μm.

The lack of synaptogenic activity in calsyntenin-1 and -2 was puzzling, so the surface expression of transfected proteins was assayed in COS cells. This revealed that Myc-C1-CFP and Myc-C2-CFP are expressed at much lower levels on COS cell surfaces compared to Myc-C3-CFP, suggesting that perhaps a lack of surface protein may account for the inability to induce presynaptic differentiation (Figure 2.3, c and d). In an attempt to increase surface expression, extracellular only versions were made of all three calsyntenin family members, in which the majority of the extracellular domain was fused to the transmembrane domain of the non-neuronal protein CD8, followed by an intracellular CFP domain. Similar to full length calsyntenin-3, the extracellular version (Myc-C3EX-CD8-CFP) induced presynaptic clustering of synapsin in contacting axons; therefore, the intracellular domain is not required for this activity in co-culture (Figure 2.3, a and b). However, despite being

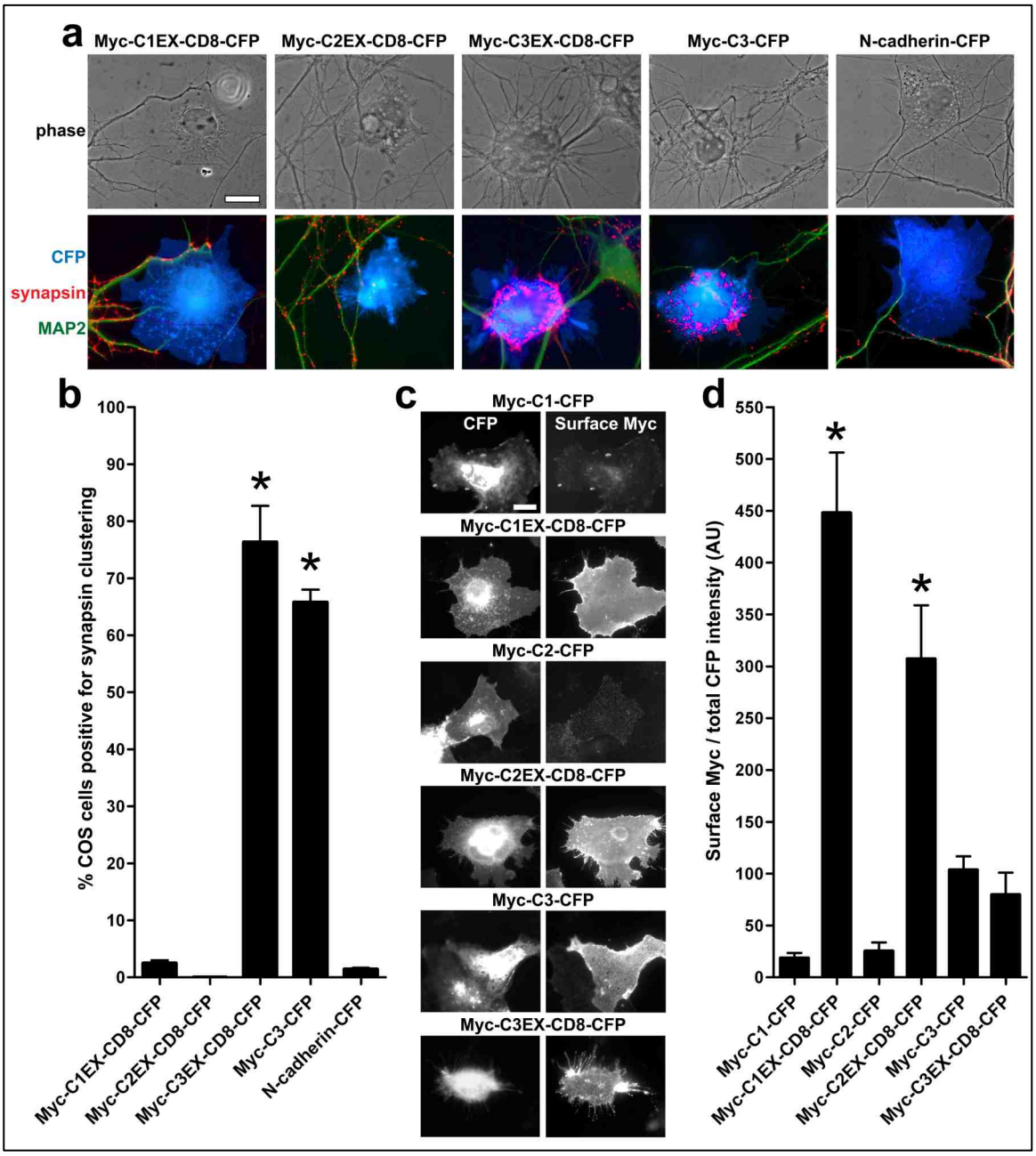
a
Myc-C1EX-CD8-CFP
Myc-C2EX-CD8-CFP
Myc-C3EX-CD8-CFP
Myc-C3-CFP
N-cadherin-CFP
phase
CFP
synapsin
MAP2
b
% COS cells positive for synapsin clustering
100
90
80
70
60
50
40
30
20
10
0
*
*
Myc-C1EX-CD8-CFP
Myc-C2EX-CD8-CFP
Myc-C3EX-CD8-CFP
Myc-C3-CFP
N-cadherin-CFP
c
Myc-C1-CFP
CFP
Surface Myc
Myc-C1EX-CD8-CFP
Myc-C2-CFP
Myc-C2EX-CD8-CFP
Myc-C3-CFP
Myc-C3EX-CD8-CFP
d
Surface Myc / total CFP intensity (AU)
550
500
450
400
350
300
250
200
150
100
50
0
*
*
Myc-C1-CFP
Myc-C1EX-CD8-CFP
Myc-C2-CFP
Myc-C2EX-CD8-CFP
Myc-C3-CFP
Myc-C3EX-CD8-CFP

Figure 2.3: An Extracellular-only, Surface-expressed Construct of Calsyntenin-3, but not Calsyntenin-1 and -2, is Active in Co-culture
(a) Truncations of all three calsyntenins were made containing only the extracellular domains, fused to the transmembrane domain of CD8. The extracellular version of calsyntenin-3 (Myc-C3EX-CD8-CFP) retains the same presynaptic protein clustering ability in COS-neuron co-cultures as wild type calsyntenin-3 (Myc-C3-CFP) (compare third column to fourth column). Extracellular versions of calsyntenin-1 and -2 (Myc-C1EX-CD8-CFP and Myc-C2EX-CD8-CFP, respectively) do not cluster synapsin in co-culture (first and second columns), similar to full-length forms. N-cadherin-CFP was used as a negative control (last column). Bottom row shows combined images of synapsin (red), dendrites by MAP2 staining (green) and transfected cells by CFP (blue). (b) Quantitation of synapsin clustering ability in co-culture of the extracellular versions of calsyntenins, by % of transfected COS cells positive for synapsin clustering without MAP2. ANOVA, $P < 0.0001$, n ≥ 3 experiments counting ≥ 100 cells per experiment; *$P < 0.001$ compared to N-cadherin-CFP by post-hoc Bonferroni test. (c) Surface expression of constructs in COS cells was assayed to determine if inactivity in co-culture was due to low surface expression. Representative images of COS expressing the indicated constructs are shown, with transfection indicated by CFP signal (left column) and surface expression measured by surface Myc staining (right column). (d) Quantitation of surface expression in COS cells, normalized to Myc-C3-CFP. Full length calsyntenin-1 and -2 were expressed on the surface of COS cells at a lower level than calsyntenin-3. However, extracellular versions of calsyntenin-1 and -2 were expressed at much higher levels on the surface, but were still not active in co-culture (shown in (a) and (b)). ANOVA, $P < 0.0001$, n ≥ 10 cells each in two independent experiments; *$P < 0.001$ compared to Myc-C3-CFP by post-hoc Bonferroni test. Results are expressed as mean ± SEM. Scale bars are 20 μm.

expressed at very high levels on the surface of COS cells (Figure 2.3, c and d), neither Myc-C1EX-CD8-CFP nor Myc-C2EX-CD8-CFP showed any induction of presynaptic specializations in co-cultures (Figure 2.3, a and b). To test for the possibility that calsyntenin-1 and -2 are synaptogenic in other neuron types but not in hippocampal cultures, Myc-C1-CFP and Myc-C2-CFP were also tested in cortical co-cultures (not shown). As no presynaptic induction was observed in cortical cultures either, it was concluded that, at least in hippocampal and cortical cultured neurons, calsyntenin-1 and -2 do not share the synaptogenic activity of calsyntenin-3. Therefore, all further analysis was restricted to calsyntenin-3 only.

In order to more specifically quantify the synaptogenic activity of calsyntenin-3, co-cultures were stained for both synapsin and the axonal marker tau, and the intensity and area of both markers was measured over transfected COS cells (Figure 2.4, a). Compared to the negative control mCFP, both Myc-C3-CFP and

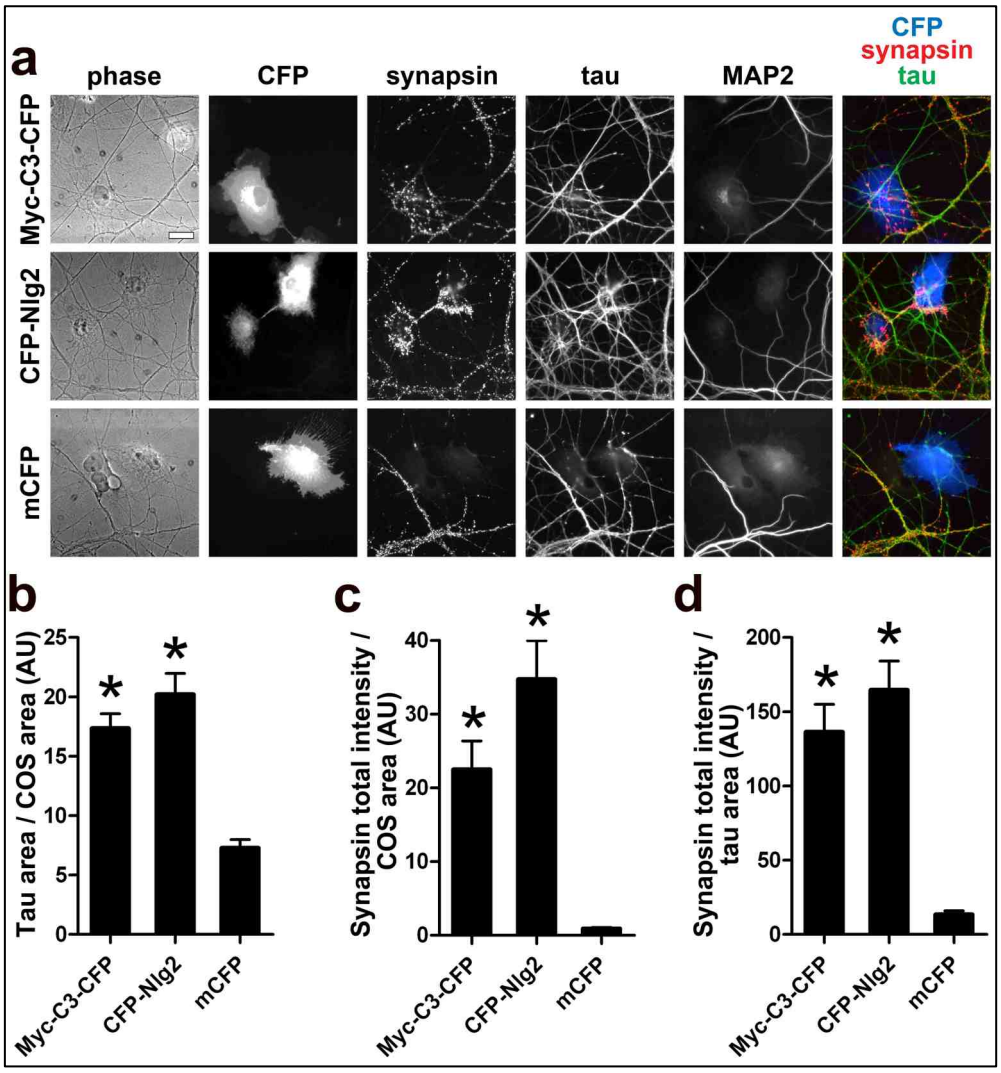

Figure 2.4: Calsyntenin-3 Increases Axon Contact, but Increases Synapsin Clustering to a Much Greater Extent

(a) COS cells expressing calsyntenin-3 (Myc-C3-CFP), neuroligin-2 as a positive control (CFP-Nlg2) or membrane-bound CFP (mCFP) as a negative control were co-cultured with hippocampal neurons. Co-culture slides were fixed and stained for the presynaptic marker synapsin (third column), the axonal marker tau (fourth column), and the dendritic marker MAP2 (fifth column). Sixth column shows combined images for Myc-C3-CFP (top), CFP-Nlg2 (middle) and mCFP (bottom) with transfected cells shown by CFP (blue), synapsin (red) and axons marked by tau (green). Scale bar is 20 μm. (b) Quantitation of tau signal over transfected COS cells in the co-culture assay. Both calsyntenin-3 and neuroligin-2 increase tau area compared to control mCFP. ANOVA, $P < 0.0001$, n = 10 cells each in three independent experiments; *$P < 0.001$ compared to mCFP by post-hoc Bonferroni test. (c) Quantitation of synapsin clustering over transfected COS cells in the co-culture assay. Both calsyntenin-3 and neuroligin-2 increase synapsin total intensity compared to control mCFP. ANOVA, $P < 0.0001$, n = 10 cells each from three independent experiments; *$P < 0.001$ compared to mCFP by post-hoc Bonferroni test. (d) Quantitation of synapsin clustering over transfected COS cells in the co-culture assay, normalized by tau contact. Both calsyntenin-3 and neuroligin-2 increase synapsin total intensity compared to control mCFP even when the increase in axon contact is taken into account. ANOVA, $P < 0.0001$, n = 10 cells each from three independent experiments; *$P < 0.001$ compared to mCFP by post-hoc Bonferroni test.

CFP-neuroligin2 (CFP-Nlg2) expressing COS cells showed increased axon contact measured by tau area (Figure 2.4, b). As expected, both also induced large increases in synapsin total intensity (Figure 2.4, c). These large increases in synapsin were still observed even when data was normalized to account for the increase in tau area (Figure 2.4, d), demonstrating that calsyntenin-3, like neuroligin-2, is a strong inducer of presynaptic differentiation and doesn't simply increase axon contact.

2.3.3 Domain Analysis Shows that a Membrane-anchored Extracellular Domain of Calsyntenin-3 is Necessary and Sufficient for Synaptogenic Activity

In order to determine which domains of calsyntenin-3 are required for synaptogenic activity in co-culture assays, a number of deletion mutants were cloned (Figure 2.5, a). Since calsyntenin-3 can be cleaved in the extracellular domain to produce a large secreted portion, one objective was to determine if calsyntenin-3 must be anchored to the membrane to exert synaptogenic effects, or if it can act as a secreted diffusible factor. Thus, comparisons were made between a version lacking the intracellular domain only (Myc-C3EXTM-CFP), a version with a small deletion flanking the reported primary cleavage site (PCS) (Araki et al., 2004; Hata et al., 2009), (Myc-C3ΔPCS-CFP), a version lacking only an extracellular juxtamembrane region and the calsyntenin-3 transmembrane domain, in which the extracellular domain is anchored to the membrane via the transmembrane of CD8 (Myc-C3EX-CD8-C3IN), a version lacking a juxtamembrane extracellular region, the TM and the intracellular domain, anchored to the membrane by CD8 TM (Myc-C3EX-CD8-CFP), a version lacking any transmembrane anchor (Myc-C3secreted),

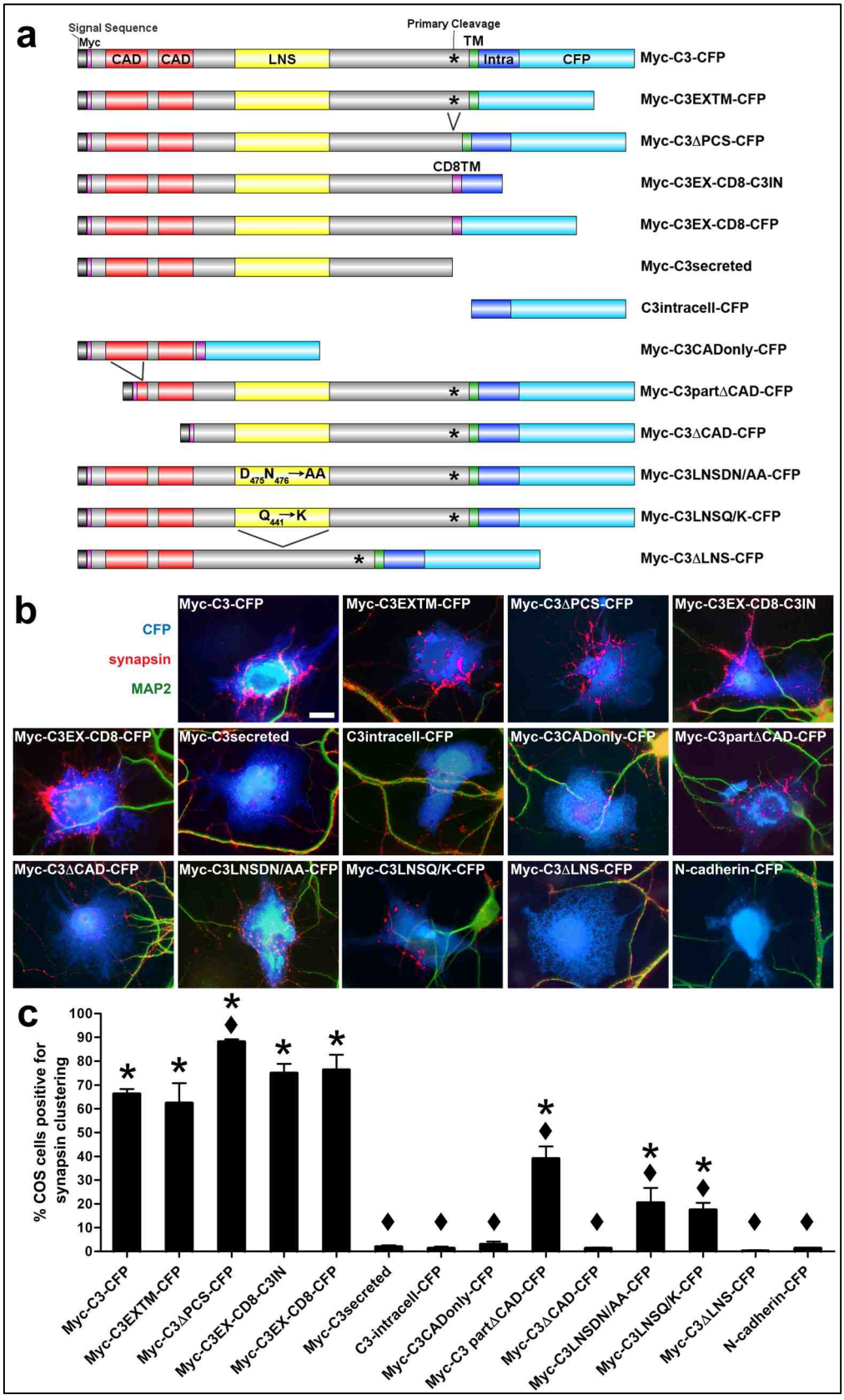
a
Signal Sequence
Myc
Primary Cleavage
TM
CAD
CAD
LNS
Intra
CFP
Myc-C3-CFP
Myc-C3EXTM-CFP
Myc-C3ΔPCS-CFP
CD8TM
Myc-C3EX-CD8-C3IN
Myc-C3EX-CD8-CFP
Myc-C3secreted
C3intracell-CFP
Myc-C3CADonly-CFP
Myc-C3partΔCAD-CFP
Myc-C3ΔCAD-CFP
D475N476→AA
Myc-C3LNSDN/AA-CFP
Q441→K
Myc-C3LNSQ/K-CFP
Myc-C3ΔLNS-CFP
b
CFP
synapsin
MAP2
Myc-C3-CFP
Myc-C3EXTM-CFP
Myc-C3ΔPCS-CFP
Myc-C3EX-CD8-C3IN
Myc-C3EX-CD8-CFP
Myc-C3secreted
C3intracell-CFP
Myc-C3CADonly-CFP
Myc-C3partΔCAD-CFP
Myc-C3ΔCAD-CFP
Myc-C3LNSDN/AA-CFP
Myc-C3LNSQ/K-CFP
Myc-C3ΔLNS-CFP
N-cadherin-CFP
c
% COS cells positive for synapsin clustering
100
90
80
70
60
50
40
30
20
10
0
Myc-C3-CFP
Myc-C3EXTM-CFP
Myc-C3ΔPCS-CFP
Myc-C3EX-CD8-C3IN
Myc-C3EX-CD8-CFP
Myc-C3secreted
C3-intracell-CFP
Myc-C3CADonly-CFP
Myc-C3 partΔCAD-CFP
Myc-C3ΔCAD-CFP
Myc-C3LNSDN/AA-CFP
Myc-C3LNSQ/K-CFP
Myc-C3ΔLNS-CFP
N-cadherin-CFP

Figure 2.5: A Membrane-anchored Extracellular Domain of Calsyntenin-3 is Necessary and Sufficient for Presynaptic Induction in Co-culture
(a) Representation of the wild type rat calsyntenin-3 construct and various domain deletion mutants. Wild type calsyntenin-3 was tagged at the N-terminus with a Myc tag. The protein contains two cadherin repeats (CAD) (in red) and one Laminin A, Neurexins, and Sex hormone-binding protein domain (LNS) (in yellow), followed by a primary extracellular cleavage site (*), a transmembrane domain (TM) (in green), and a short intracellular domain (Intra) (in dark blue). The intracellular domain was fused to cyan fluorescent protein (CFP) (in cyan) to visualize expression using fluorescence microscopy. In some deletion constructs, the wild type transmembrane domain was replaced with CD8 transmembrane domain (CD8TM) (in purple). Please see text for more details. (b) Representative images of domain analysis for calsyntenin-3 co-culture activity. Each panel is a combined image showing the indicated construct transfected in a COS cell (CFP shown in blue), contacting dendrites shown by MAP2 (green), and presynaptic clustering shown by synapsin puncta (red). Constructs without CFP tags were co-transfected with plain CFP for visualization. Scale bar is 20 μm. (c) Quantitation of domain analysis, by % of transfected COS cells positive for synapsin clustering without MAP2 for each indicated construct. Constructs containing the extracellular domain retained presynaptic protein clustering activity, and a construct containing only the intracellular domain was inactive. However, a "secreted" form lacking a transmembrane domain was not active. Therefore, the extracellular portion of calsyntenin-3 must be tethered to the membrane for activity, but the intracellular domain is not required. A construct containing only the CAD repeats was not active. Partial deletion of the first cadherin domain (Myc-C3partΔCAD-CFP), and point mutations in the LNS domain (Myc-C3LNSDN/AA-CFP; Myc-C3LNSQ/K-CFP) decreased co-culture activity but did not completely abolish it, and full deletion of either the cadherin or the LNS domains abolished activity. Therefore, both the CAD repeats and the LNS domain in the extracellular region are required for presynaptic protein clustering in co-culture. ANOVA, $P < 0.0001$, n ≥ 3 experiments counting ≥ 100 cells per experiment; *$P < 0.001$ compared to N-cadherin-CFP by post-hoc Bonferroni test. ♦ $P < 0.001$ compared to Myc-C3-CFP by post-hoc Bonferroni test. Results are expressed as mean ± SEM.

and a version consisting of only the short intracellular domain of calsyntenin-3 (C3intracell-CFP). Expression of these constructs in co-cultures with neurons showed that all versions containing a membrane-anchored extracellular domain induce presynaptic synapsin clustering, while the intracellular domain was not necessary for this activity (Figure 2.5, b and c). The secreted version was not positive in co-culture, indicating that calsyntenin-3 acts by clustering presynaptic proteins locally rather than acting as a diffusible promoter of synaptogenesis. Domain-specific deletions were also constructed to remove the cadherin domains or the LNS domain. In addition, two LNS domain point-mutations were generated: the Myc-C3LNSDN/AA-CFP mutant results in the disruption of two amino acids predicted to be similar to two adjacent aspartic acid residues important

for calcium binding in the LNS domain of β-neurexins (Arac et al., 2007; Chen et al., 2008; Fabrichny et al., 2007; Graf et al., 2006; Rudenko et al., 2001; Shen et al., 2008), while the Myc-C3LNSQ/K-CFP is designed to mimic a point mutation associated with learning defects isolated in the *C. elegans* calsyntenin ortholog, CASY-1 (Ikeda et al., 2008). Testing these mutants in co-culture revealed that partial deletion of the first cadherin domain (Myc-C3partΔCAD-CFP) and both LNS point mutations decreased the synaptogenic activity, but did not abolish it (Figure 2.5, b and c). Furthermore, deletion of either the LNS domain (Myc-C3ΔLNS-CFP) or both cadherin domains (Myc-C3ΔCAD-CFP) resulted in complete loss of synaptogenic activity, as did a version consisting of only the cadherin repeats (Myc-C3CADonly-CFP). These results show that both the cadherin and LNS domains are necessary for induction of presynaptic differentiation in culture.

In order to further characterize the various deletion constructs, cleavage patterns and surface expression were assayed in COS cells. The presence of cleavage products was assayed in the COS cell lysates and in media by western blotting against the N-terminal Myc-tag, which would recognize full length Myc-calsyntenin-3-CFP (~130 kDa) and the large cleaved extracellular fragment (~110 kDa). Blots show that for constructs which are predicted to contain the primary cleavage site, such as Myc-C3-CFP and Myc-C3EXTM-CFP, both a full length and cleaved product is present in cell lysates, and a cleavage product is detected in the media (Figure 2.6, a). On the other hand, Myc-C3EX-CD8-CFP, which is fused to CD8 TM domain upstream of the cleavage site, is only seen in full length form and not detected in the media, and Myc-C3secreted is found abundantly in the media.

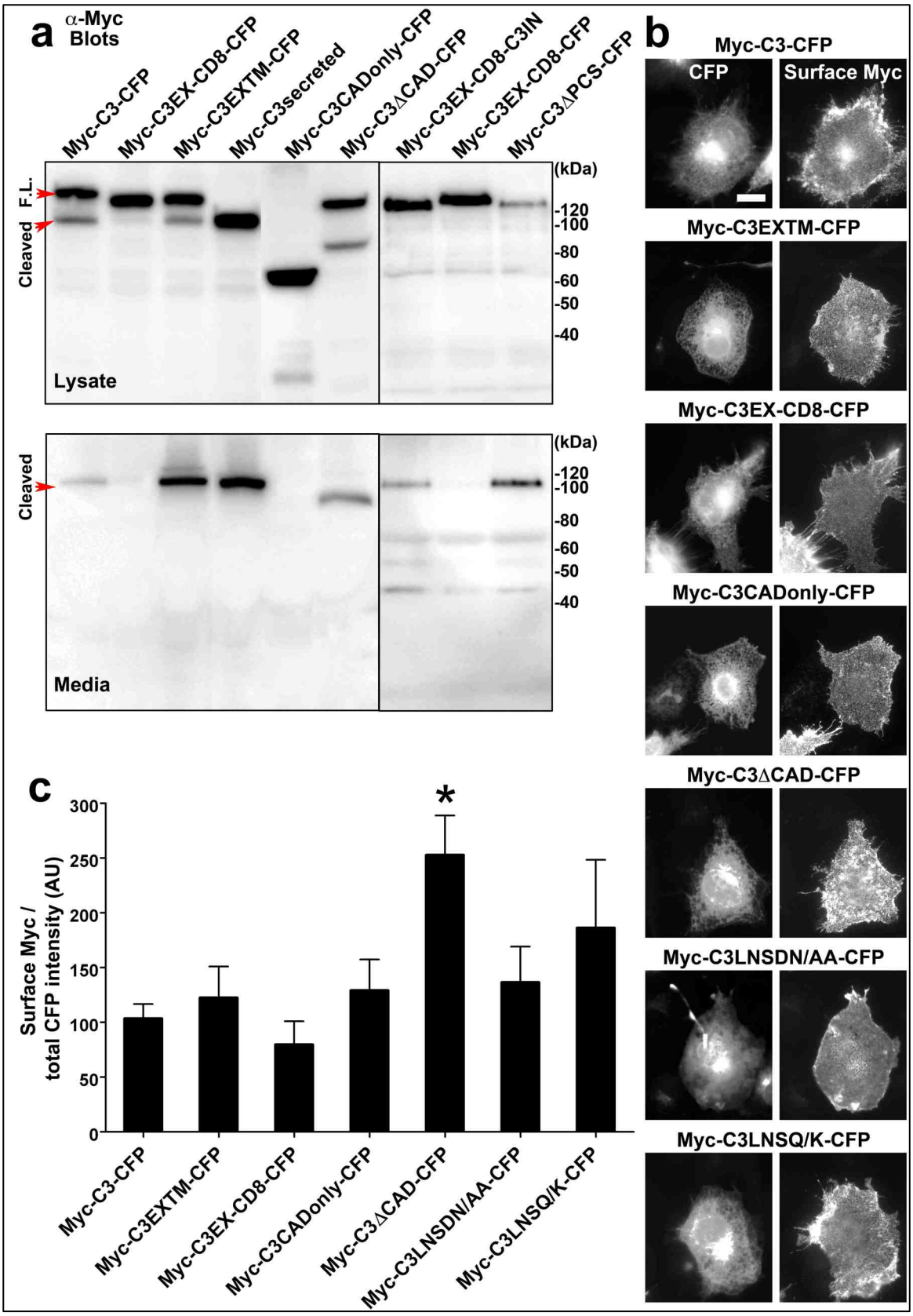
a
α-Myc Blots
Myc-C3-CFP
Myc-C3EX-CD8-CFP
Myc-C3EXTM-CFP
Myc-C3secreted
Myc-C3CADonly-CFP
Myc-C3ΔCAD-CFP
Myc-C3EX-CD8-C3IN
Myc-C3EX-CD8-CFP
Myc-C3ΔPCS-CFP
F.L.
Cleaved
(kDa)
-120
-100
-80
-60
-50
-40
Lysate
Cleaved
(kDa)
-120
-100
-80
-60
-50
-40
Media
b
Myc-C3-CFP
CFP
Surface Myc
Myc-C3EXTM-CFP
Myc-C3EX-CD8-CFP
Myc-C3CADonly-CFP
Myc-C3ΔCAD-CFP
Myc-C3LNSDN/AA-CFP
Myc-C3LNSQ/K-CFP
c
Surface Myc / total CFP intensity (AU)
300
250
200
150
100
50
0
*
Myc-C3-CFP
Myc-C3EXTM-CFP
Myc-C3EX-CD8-CFP
Myc-C3CADonly-CFP
Myc-C3ΔCAD-CFP
Myc-C3LNSDN/AA-CFP
Myc-C3LNSQ/K-CFP

Figure 2.6: Characterization of Calsyntenin-3 Deletion Constructs
(a) Wild type calsyntenin-3 and various domain deletion constructs were expressed in COS cells. To assay for cleavage of the extracellular domain, both cell lysate (top blot) and conditioned media (bottom blot) were collected and run on an SDS-PAGE gel. Black boxes represent gels that were run separately. Western blotting with an anti-Myc antibody was used to detect both full length (~130 kDa) and cleaved N-terminal (~100 kDa) products of Myc-tagged constructs. As expected, protein from constructs containing the putative primary cleavage site can be found in the cell media. Myc-C3EX-CD8-CFP (second lane) is not found in media and thus not cleaved. However, Myc-C3ΔPCS-CFP (last lane), which contains only a small extracellular region deletion flanking the putative primary cleavage site, is still found in conditioned cell media, suggesting the consensus sequence for cleavage is perhaps quite non-specific. In addition, Myc-C3EX-CD8-C3IN (seventh lane) is also found in cell media, suggesting that perhaps the intracellular region of calsyntenin is important for proper targeting to initiate cleavage. (b) Surface expression of calsyntenin-3 constructs in COS cells was assayed to determine if inactivity in co-culture was due to low surface expression. Representative images of COS expressing the indicated constructs are shown, with CFP expression indicating transfection (left column), and surface Myc staining used to assess surface expression (right column). Scale bar is 20 μm. (c) Quantitation of surface expression in COS cells, normalized to Myc-C3-CFP. Of the constructs tested, none had a significant change in surface expression compared to Myc-C3-CFP, with the exception of Myc-C3ΔCAD-CFP. Since no constructs were expressed significantly less than Myc-C3-CFP, it is clear that inactivity in co-culture is not simply due to low surface expression in COS for any of these deletion mutants. ANOVA, $P < 0.0001$, $n \geq 10$ cells each in two independent experiments; $^{*}P < 0.01$ compared to Myc-C3-CFP by post-hoc Bonferroni test. Results are expressed as mean ± SEM.

These results, when considered together with the co-culture assays, confirm that calsyntenin-3 does not need to be cleaved in order to be synaptogenic (as Myc-C3EX-CD8-CFP is positive in co-culture, and is not cleaved in COS cells), and in fact needs to be anchored to the membrane (as Myc-C3secreted is in fact found in media, and is negative in co-culture). In addition, Myc-C3CADonly-CFP does not contain the primary cleavage site, and is not detected in the media, while Myc-C3ΔCAD-CFP does contain the site, and is detected in the media.

However, some results from the western analysis are puzzling. Myc-C3ΔPCS-CFP is a mutant with a very small excision (20 amino acids) that encompasses the cleavage site. However, cleavage products from this construct are detected in COS cell media, which suggest that the cleavage consensus sequence may be quite large, or, more likely, that the proteases that cleave calsyntenin-3 in COS cells are quite promiscuous. Indeed, this is likely the case as studies on extracellular

metalloproteases show that few substrates have consensus sequences for cleavage and the cleavage sites themselves can be highly variable (Black et al., 2003; White, 2003). Instead, it appears that the secondary structure of the juxtamembrane region largely determines substrate recognition (Seals and Courtneidge, 2003). Even more puzzling is the presence of Myc-C3EX-CD8-C3IN cleavage products in COS cell media. This construct is exactly the same as Myc-C3EX-CD8-CFP in the extracellular region, and only differs in that it contains the calsyntenin-3 cytoplasmic domain instead of CFP. It is possible that the majority of cleavage of calsyntenins occurs not at the plasma membrane but rather during vesicular transport or in the endoplasmic reticulum, where proteases are also found, and that the cytoplasmic domain allows for correct targeting to allow for cleavage. However, this hypothesis does not explain the fact that Myc-C3EXTM-CFP, which contains the entire extracellular and TM domains but lacks the cytoplasmic domain, is still cleaved and found in media. In addition, although ADAM10 and ADAM17 have been shown to cleave calsyntenin-3 (Hata et al., 2009), it is possible that it is also a substrate for other proteases. A number of different proteases with diverse substrate recognition requirements may explain these discrepancies, although further investigation would be required to examine this possibility.

Surface expression of a selection of deletion mutants was also assayed, with the main goal of confirming that constructs that are negative in the co-culture are in fact expressed on COS cell surfaces, and aren't simply negative due to low expression. Results showed that, with the exception of Myc-C3ΔCAD-CFP, none of the constructs tested is expressed at a significantly different level on COS cell

surfaces compared to Myc-C3-CFP (Figure 2.6, b and c). Myc-C3ΔCAD-CFP, on the other hand, is actually expressed significantly higher on COS cell surfaces than Myc-C3-CFP, thus lack of surface expression cannot account for decreased or abolished co-culture activity in any of the constructs tested.

2.3.4 Calsyntenin-3 is Synaptogenic at Excitatory and Inhibitory Synapses

Recent advances in the field of synaptogenesis have uncovered a great many cell adhesion and secreted proteins involved in synapse development at glutamatergic synapses (Siddiqui and Craig, 2011). However, GABAergic synapses have been much less studied, with neuroligin-2 being the main inhibitory postsynaptic “inductive” CAM described to date. To determine which types of hemi-presynapses calsyntenin-3 can induce, co-cultures were stained with markers for excitatory (presynaptic VGlut1 and postsynaptic PSD95) or inhibitory (presynaptic VGAT and postsynaptic gephyrin) synaptic markers (Figure 2.7, a and b). Quantitation revealed that, like neuroligin-2, calsyntenin-3 can induce clustering of both glutamatergic and GABAergic presynaptic proteins; the level of induction was approximately equal for both types of synapses (Figure 2.7, c and d).

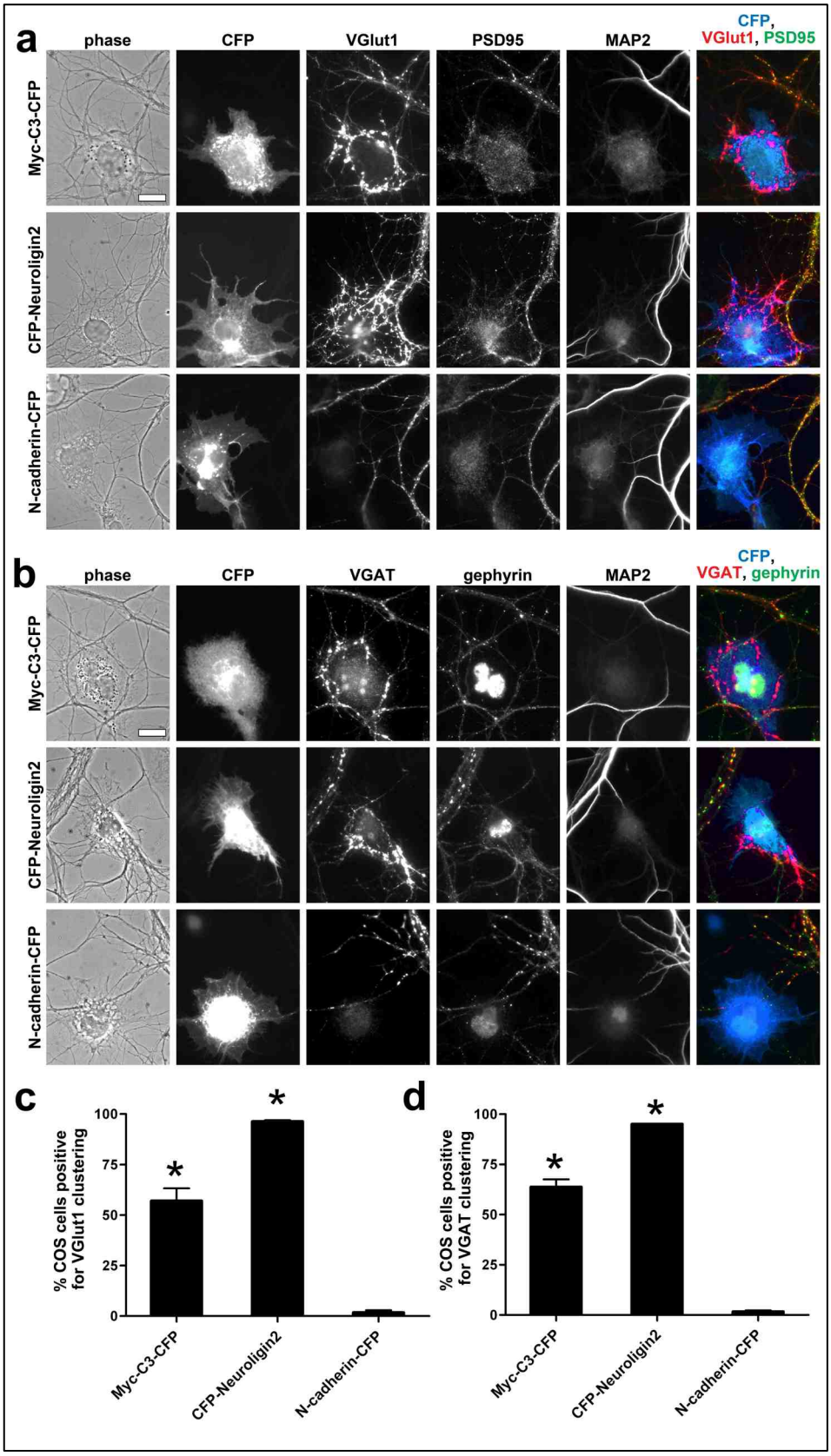
a
phase
CFP
VGlut1
PSD95
MAP2
CFP, VGlut1, PSD95
Myc-C3-CFP
CFP-Neuroligin2
N-cadherin-CFP
b
phase
CFP
VGAT
gephyrin
MAP2
CFP, VGAT, gephyrin
Myc-C3-CFP
CFP-Neuroligin2
N-cadherin-CFP
c
% COS cells positive for VGlut1 clustering
100
75
50
25
0
*
*
Myc-C3-CFP
CFP-Neuroligin2
N-cadherin-CFP
d
% COS cells positive for VGAT clustering
100
75
50
25
0
*
*
Myc-C3-CFP
CFP-Neuroligin2
N-cadherin-CFP

Figure 2.7: Calsyntenin-3 Can Induce Both Excitatory and Inhibitory Presynaptic Protein Clustering
(a) Co-culture assays stained for the excitatory synaptic markers VGlut1 (presynaptic) (third column) and PSD95 (postsynaptic) (fourth column). Myc-C3-CFP (top row), like CFP-Neuroligin2 (middle row), can induce clustering of VGlut1 over transfected COS cells without apposing PSD95. N-cadherin-CFP was used as a negative control (bottom row). The last column shows combined images for each construct with expressing COS cells shown by CFP (blue), VGlut1 (red) and PSD95 (green). (b) Co-culture assays stained for the inhibitory synaptic markers VGAT (presynaptic) (third column) and gephyrin (postsynaptic) (fourth column). Myc-C3-CFP (top row), like CFP-neuroligin2 (middle row), can induce clustering of VGAT over transfected COS cells without apposing gephyrin. N-cadherin-CFP was used as a negative control (bottom row). The last column shows combined images for each construct with expressing COS cells shown by CFP (blue), VGAT (red) and gephyrin (green). Scale bars are 20 μm. (c) Quantitation of VGlut1 clustering without apposed PSD95 over transfected COS cells for each construct. ANOVA, $P < 0.0001$, n ≥ 3 experiments counting ≥ 100 cells per experiment; *$P < 0.001$ compared to N-cadherin-CFP by post-hoc Bonferroni test. (d) Quantitation of VGAT clustering without apposed gephyrin over transfected COS cells for each construct. ANOVA, $P < 0.0001$, n ≥ 3 experiments counting ≥ 100 cells per experiment; *$P < 0.001$ compared to N-cadherin-CFP by post-hoc Bonferroni test.

2.3.5 Overexpression of Calsyntenin-3 Increases Clustering of Presynaptic Proteins

Since calsyntenin-3 increases presynaptic protein clustering when expressed in COS cells, it was hypothesized that it might have similar effects in neurons. For these experiments, wild-type calsyntenin-3 (Myc-C3-CFP) was not utilized, as it did not accumulate on neuron surfaces at a high enough level to be detected by surface Myc immunostaining. It is possible that, in the hippocampal neuron culture system, there is increased constitutive proteolytic processing or upregulation of proteases that cleave calsyntenin-3. Therefore, other deletion mutants were tested for surface expression levels when overexpressed in hippocampal neurons, and it was determined that both Myc-C3ΔPCS-CFP ("ΔPCS") and Myc-C3EX-CD8-C3IN ("EX-CD8-IN") attained high levels of accumulation on dendrite surfaces (Figure 2.8, a and d). In COS cells, both of these constructs are still cleaved, but apparently they are cleaved less than Myc-C3-CFP in the hippocampal cultures, which would allow for increased surface accumulation. On the other hand, Myc-C3EX-CD8-CFP, which

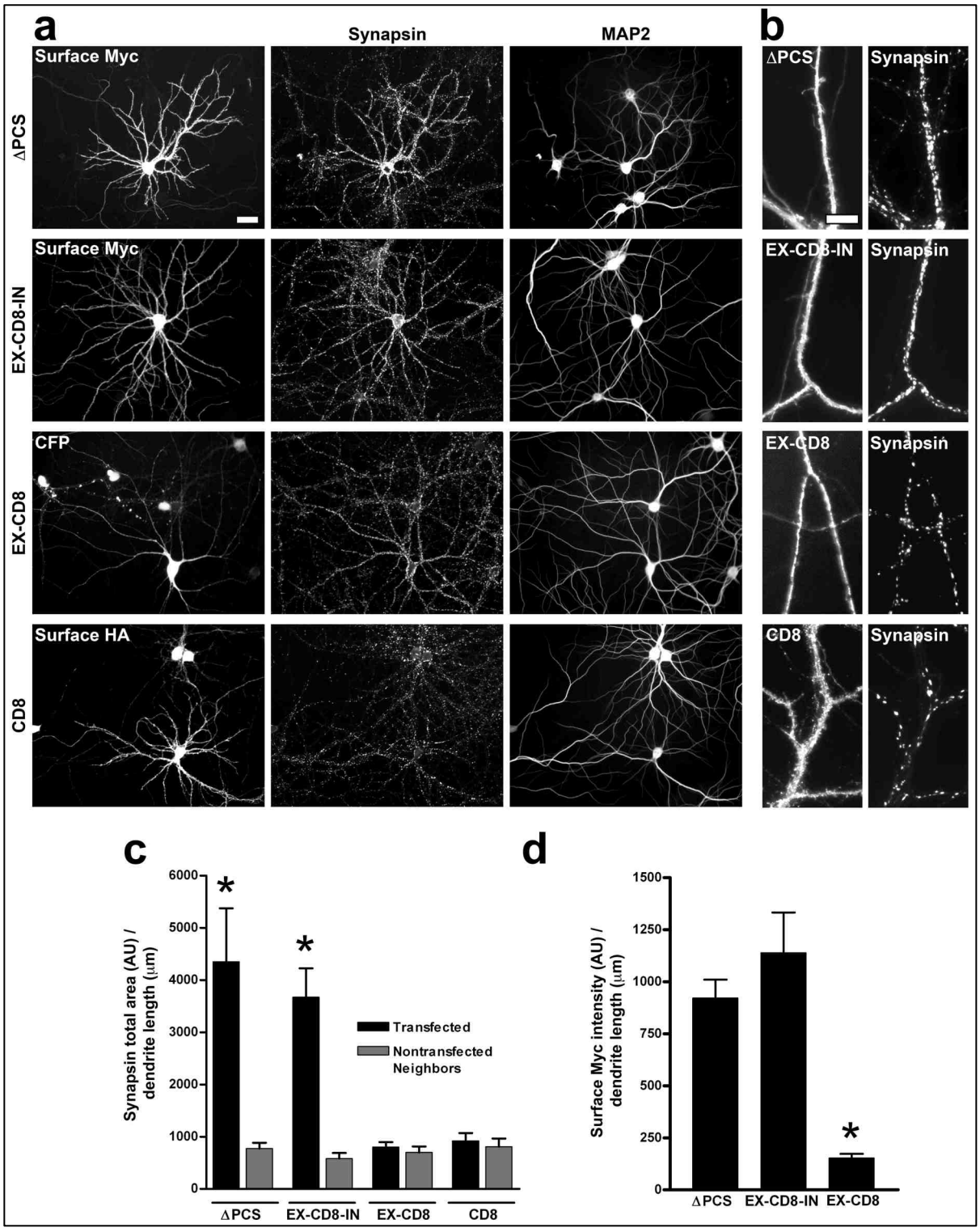
a
Synapsin
MAP2
ΔPCS
Surface Myc
EX-CD8-IN
Surface Myc
EX-CD8
CFP
CD8
Surface HA
b
ΔPCS
Synapsin
EX-CD8-IN
Synapsin
EX-CD8
Synapsin
CD8
Synapsin
c
Synapsin total area (AU) / dendrite length (μm)
6000
5000
4000
3000
2000
1000
0
*
*
Transfected
Nontransfected Neighbors
ΔPCS
EX-CD8-IN
EX-CD8
CD8
d
Surface Myc intensity (AU) / dendrite length (μm)
1500
1250
1000
750
500
250
0
*
ΔPCS
EX-CD8-IN
EX-CD8

Figure 2.8: Calsyntenin-3 Overexpression Increases Synapsin Clustering in Culture
(a and b) Cultured hippocampal neurons were transfected at 8 – 9 DIV with Myc-C3ΔPCS-CFP (ΔPCS) (top row), Myc-C3EX-CD8-C3IN (EX-CD8-IN) (second row), Myc-C3EX-CD8-CFP (EX-CD8) (third row), or HA-CD8 (CD8) as a negative control (bottom row). Neurons were stained for synapsin and MAP2 at 14 DIV. Neurons transfected with ΔPCS or EX-CD8-IN, imaged at 25X (a) and 63X (b), show an increase in synapsin when compared to non-transfected neighboring neurons (compare synapsin staining (middle column in (a) in neighbors (shown by MAP2 staining in third column) to transfected cell (shown in first column)). No change in synapsin was observed for neurons transfected with EX-CD8 or negative control CD8. Scale bar is 30 μm in (a) and 10 μm in (b). (c) Quantitation of synapsin total area in transfected neurons along with non-transfected neighboring neurons. ANOVA, $P < 0.0001$, n = 10 cells each in two independent experiments; $^*P < 0.001$ compared to CD8 in post-hoc Bonferroni test. (d) Dendritic surface expression of Myc-tagged calsyntenin-3 constructs was also measured. ΔPCS and EX-CD8-IN had similar levels of surface expression. EX-CD8 was expressed at a much lower level on the surface of dendrites, which explains the lack of effect on synapsin clustering. ANOVA, $P < 0.0001$, n = 10 cells each in two independent experiments; $^*P < 0.001$ compared to ΔPCS in post-hoc Bonferroni test. Results are expressed as mean ± SEM.

is not cleaved in COS cells, was found at very low levels on neuron surfaces and could only be easily detected via the cytoplasmic CFP signal (Figure 2.8, a and d). Therefore, initial analysis of overexpression effects was done using ΔPCS and EX-CD8-IN, with EX-CD8 and HA-CD8 as negative controls. Overexpression of either ΔPCS or EX-CD8-IN resulted in large increases in synapsin onto transfected dendrites compared to dendrites of nontransfected neighboring neurons (Figure 2.8, a-c). Close inspection of transfected dendrites at 63 X magnification showed that the increased synapsin was still present in clusters and did not fill axons (Figure 2.8, b). Synapsin clustering was not changed in EX-CD8 or CD8 transfected dendrites compared to nontransfected neighbors.

Given the increase in synapsin clustering, it seemed likely that calsyntenin-3 overexpression in neurons could also affect the clustering of excitatory and inhibitory presynaptic proteins. Myc-C3ΔPCS-CFP was used for this analysis as it had the highest effect on synapsin clustering in the initial analysis, and also because it is the construct bearing closest resemblance to wild type calsyntenin-3. Overexpression of

Myc-C3ΔPCS-CFP increased the clustering of both excitatory VGlut1 and inhibitory VGAT onto transfected dendrites compared to control transfection of soluble CFP (solCFP) (Figure 2.9, a, b, e, g). Interestingly, this increase was accompanied by a dispersal of postsynaptic PSD95 and gephyrin (Figure 2.9, c, d, f, h). Neuroligin-2 overexpression also induced an increase in presynaptic clustering with a dispersal of postsynaptic clustering, which is consistent with previous reports (Graf et al., 2004). It is likely that the dispersal of postsynaptic proteins is due to the very high level of dendritic neuroligin-2 (or calsyntenin-3) expression that fills dendrites; this essentially mislocalizes the protein from synaptic sites and increases extrasynaptic expression, thus resulting in concomitant mislocalization of other postsynaptic proteins (in this case, PSD95 and gephyrin). These results suggest that, like neuroligin-2, postsynaptic calsyntenin-3 may be able to mediate intracellular signaling to affect postsynaptic protein clustering.

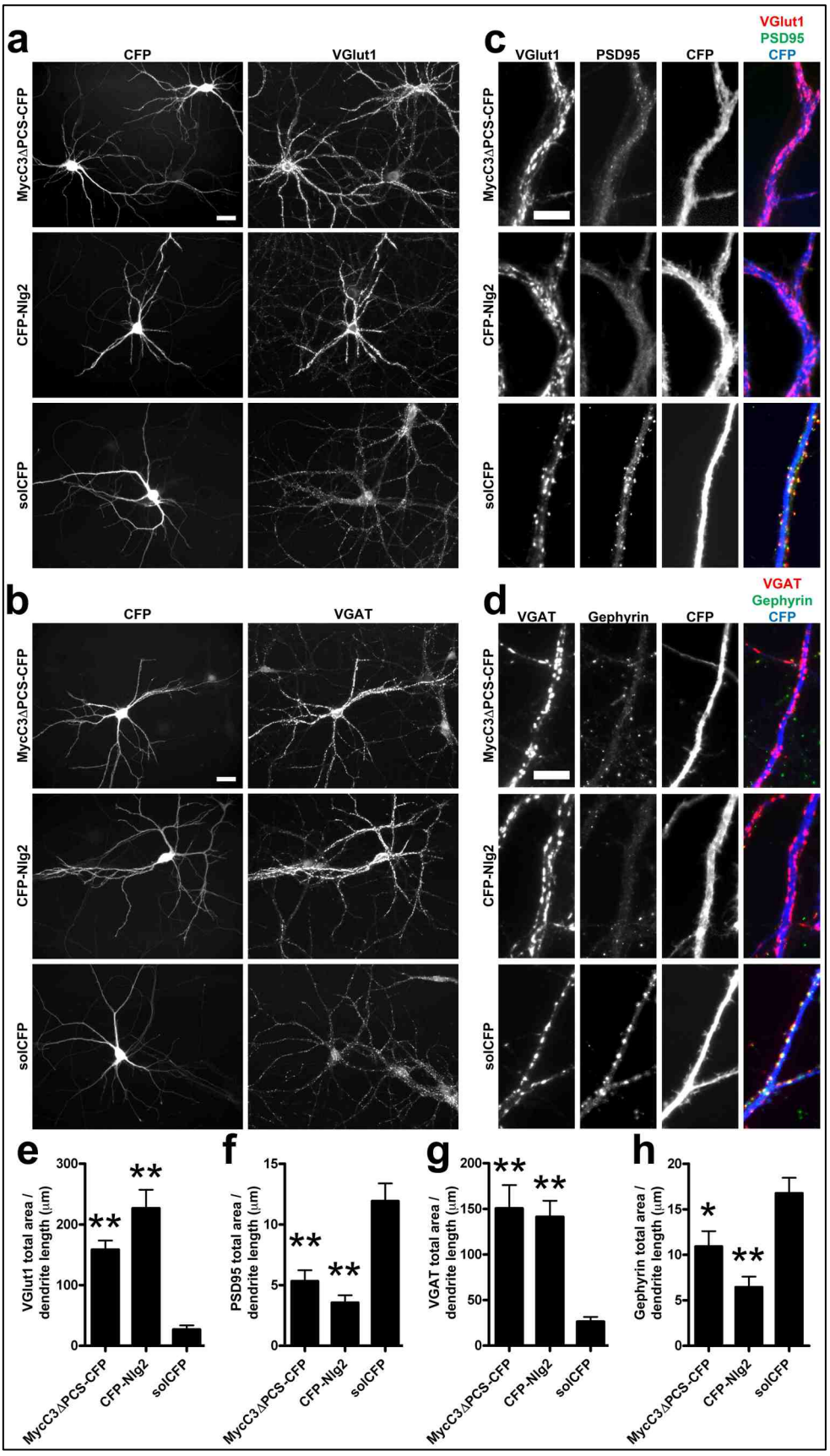
a
CFP
VGlut1
MycC3ΔPCS-CFP
CFP-Nlg2
solCFP
c
VGlut1
PSD95
CFP
VGlut1
PSD95
CFP
b
CFP
VGAT
d
VGAT
Gephyrin
CFP
VGAT
Gephyrin
CFP
e
VGlut1 total area / dendrite length (μm)
f
PSD95 total area / dendrite length (μm)
g
VGAT total area / dendrite length (μm)
h
Gephyrin total area / dendrite length (μm)
MycC3ΔPCS-CFP
CFP-Nlg2
solCFP

Figure 2.9: Calsyntenin-3 Overexpression Increases Excitatory and Inhibitory Presynaptic Clustering in Culture
(a and b) Cultured hippocampal neurons were transfected at 8 – 9 DIV with Myc-C3ΔPCS-CFP (top rows), CFP-neuroligin2 (CFP-Nlg2) as a positive control (middle rows), or soluble CFP (solCFP) as a negative control (bottom rows). Neurons were stained for synaptic markers at 14 DIV. Transfected neurons (first column) imaged at 25X show an increase in both VGlut1 (a) and VGAT (b) (second column) when transfected with Myc-C3ΔPCS-CFP or CFP-Nlg2, but not with negative control solCFP. Scale bars are 30 µm. (c, d) Dendrites imaged at 63X reveal that this increase in presynaptic clustering is accompanied by a dispersal of postsynaptic PSD95 (c) or gephyrin (d) (second column). Last column shows combined images with the presynaptic marker (VGlut1 or VGAT) in red, postsynaptic marker (PSD95 or gephyrin) in green and transfected dendrite in blue. Scale bars are 10 µm. (e - h) Quantitation of total cluster area per dendrite length for VGlut (e), PSD95 (f), VGAT (g) and gephyrin (h) in neurons overexpressing Myc-C3ΔPCS-CFP or controls. ANOVA, $P < 0.0001$, n = 10 each in two independent experiments; $^{**}P < 0.001$, $^{*}P < 0.05$ compared to solCFP in post-hoc Bonferroni test for (e), (f), (g) and (h). Results are expressed as mean ± SEM.

2.3.6 Calsyntenin-3 Binds to Neurexin-1α

In the case of neuroligin-2, its presynaptic binding partner neurexin mediates the trans-synaptic induction of presynaptic protein clustering (Graf et al., 2004). Given the recent discovery of a number of other postsynaptic, synaptogenic partners for neurexins (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010; Uemura et al., 2010), it seemed possible that calsyntenin-3 may also act through neurexins. To test this hypothesis, a calsyntenin-3 fusion protein was generated containing the extracellular portion of calsyntenin-3 fused to the fragment crystallizable (Fc) portion of human immunoglobulin (Clstn3-Fc). Binding of Clstn3-Fc to COS cells expressing HA-neurexin-1α was assayed, and revealed that Clstn3-Fc specifically binds to neurexin-1α (Figure 2.10, a). Further assays with a range of concentrations showed that saturated binding was achieved and Scatchard analysis indicated a high binding affinity of 43.7 ± 10.0 nM (Figure 2.10, b).

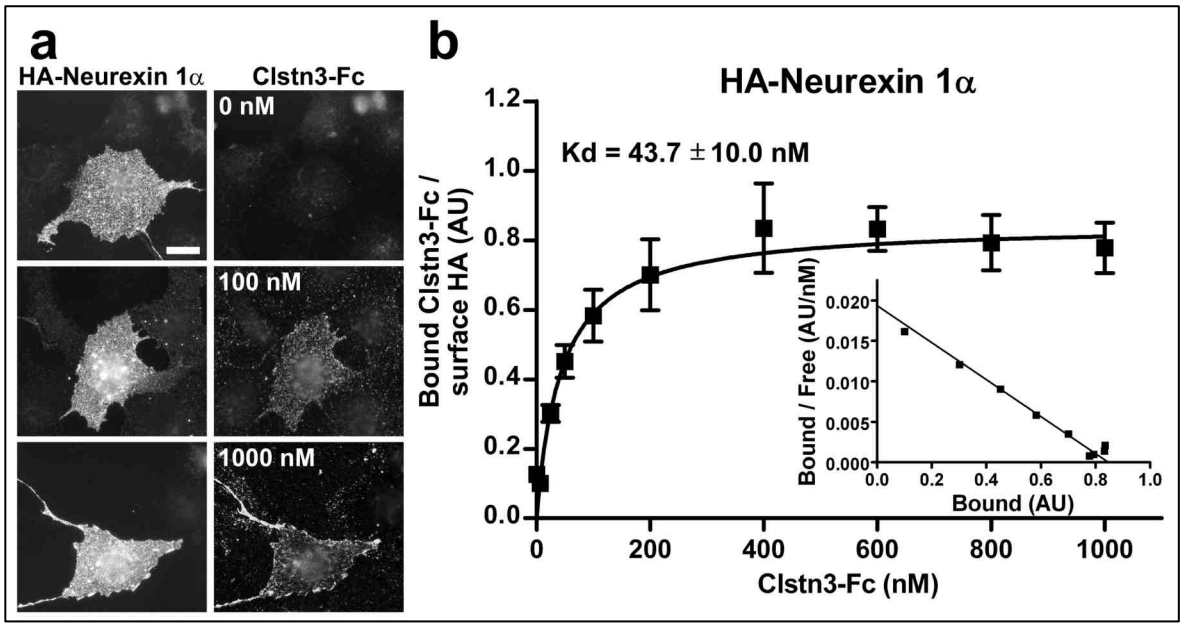

Figure 2.10: Calsyntenin-3 Binds with High Affinity to Neurexin1α
(a) Binding of calsyntenin-3-Fc protein (Clstn3-Fc) to COS cells expressing HA-tagged Neurexin1α (HA-Neurexin1α). Surface HA stain to identify transfected COS cells is shown in the left column, and binding of Clstn3-Fc protein at the indicated concentrations is shown in the right column. Scale bar is 20 μm. (b) By Scatchard analysis, affinity of binding of Clstn3-Fc to HA-Neurexin1α was estimated at 43.7 ± 10.0 nM (n ≥ 15 cells each in two independent experiments).

2.4 Discussion

In this study, an unbiased expression screen was used to isolate calsyntenin-3 as a novel synaptogenic protein (Figure 2.1). Comparison with the other two family members, calsyntenin-1 and -2, showed that calsyntenin-3 is unique in its ability to induce presynaptic specializations in the co-culture assay (Figures 2.2 and 2.3). Calsyntenin-3 can cluster a number of different presynaptic proteins in contacting axons, and induced presynaptic terminals appear to be competent for neurotransmitter release (Figure 2.2). Like other strong inducers of presynaptic differentiation such as neuroligin-2, calsyntenin-3 increases presynaptic protein clustering to a greater extent than it increases axon contact (Figure 2.4). Domain analysis revealed that both the cadherin and LNS domains are important for

synaptogenic activity in co-culture and the extracellular domain must be membrane-anchored to retain activity, while the intracellular domain was dispensable (Figures 2.5 and 2.6). This suggests that calsyntenin induces presynaptic differentiation through direct trans-synaptic adhesion rather than via activation of intracellular signaling pathways or secretion of the extracellular domain. In co-cultures, calsyntenin-3 can cluster both excitatory and inhibitory presynaptic proteins (Figure 2.7). Overexpression in neurons showed that calsyntenin-3 increases clustering of synapsin, VGlut1 and VGAT onto transfected dendrites, again suggesting activity at both excitatory and inhibitory synapses (Figures 2.8 and 2.9). Remarkably, initial results also show that calsyntenin-3 binds neurexin-1α at high affinity (Figure 2.10). Taken together, this study identifies calsyntenin-3 as a new potent inducer of synaptogenesis and suggests that it acts through trans-synaptic adhesion via binding to neurexins.

It is interesting that neither calsyntenin-1 nor calsyntenin-2 induce presynaptic specializations in co-cultures, despite sharing highly similar extracellular domains with calsyntenin-3. All three calsyntenins are expressed in the hippocampus in differential patterns, although overall expression of calsyntenin-2 is much lower than calsyntenin-1 or -3 (Hintsch et al., 2002). However, calsyntenin-1 has recently been shown to function as a cargo docking protein for kinesin, so perhaps it has a very different main function in neurons than calsyntenin-3 (Araki et al., 2007; Konecna et al., 2006; Ludwig et al., 2009; Steuble et al., 2010; Vagnoni et al., 2011). In fact, the first report of calsyntenin binding to the kinesin light chain showed that binding to calsyntenin-3 was much weaker than that to calsyntenin-1 or -2; this may be

explained by the divergence in the calsyntenin-3 cytoplasmic domain compared to the other calsyntenin family members (Konecna et al., 2006). Further studies will be required to determine if calsyntenin-1 and -2 play roles in synaptogenesis or synaptic maturation in other areas of the brain. Ultimately, the best way to approach this issue is through the generation of null calsyntenin mice.

Another issue that warrants further investigation is the impact that proteolytic processing has on the synaptogenic activities of calsyntenin-3. These results show that the calsyntenin-3 extracellular domain must be membrane-anchored in order to exert synaptogenic effects, as the secreted form showed no induction of presynaptic protein clustering. Thus, regulation of calsyntenin-3 cleavage is likely to play a major role in controlling synaptogenic effects. It is possible that proteolytic processing is regulated in a spatial and/or temporal manner during development, which could influence when and where in the brain calsyntenin-3 contributes to synaptogenesis. Cleavage could also be regulated by neuronal activity, which could provide another level of control.

Regulated cleavage of synaptic transmembrane proteins is beginning to emerge as a common theme in synaptic development (Ethell and Ethell, 2007). For example, ADAM10 can cleave N-cadherins; cleavage directly affects adhesive properties of cells and also redistributes β-catenin from the cell surface to the cytoplasmic pool, thereby influencing the expression of β-catenin target genes (Reiss et al., 2005; Uemura et al., 2006). L1, a member of the Ig superfamily involved in neurite outgrowth, is also cleaved by ADAM10 and ADAM17 by both constitutive and regulated mechanisms, which results in increased neurite outgrowth

(Maretzky et al., 2005). Ectodomain shedding of CALEB, a transmembrane protein important for maintaining normal release probability early in development, is induced in response to neuronal activity and results in exposure of an EGF domain which may be needed for binding (Juttner et al., 2005). The full-length neuronal pentraxin receptor (NPR) associates with AMPA receptors and enhances synaptogenesis, but activation of metabotropic glutamate receptors induces ADAM17-mediated cleavage of NPR and induces accumulation of cleaved NPR and AMPA receptors in endosomes. This removal of AMPA receptors from the synaptic membrane is required for metabotropic glutamate receptor-dependent LTD (Cho et al., 2008). Thus, ectodomain shedding can activate or inactivate trans-synaptic interactions. Alternatively, cleavage releases an active diffusible factor. The matter is even more complex as regulated ectodomain shedding is often followed by intramembrane cleavage and release of a small intracellular domain (ICD). The ICD is often involved in activating second messenger cascades or regulating gene transcription (Lee et al., 2008b; Reiss and Saftig, 2009). In the case of calsyntenin-1, this ICD can suppress gene transactivation mediated by the APP ICD (Araki et al., 2004). The fact that APP, a well-characterized substrate for ADAMs, also promotes synaptogenesis (Wang et al., 2009) and that calsyntenins and APP appear to undergo coordinated metabolism (Araki et al., 2004; Araki et al., 2003) raises intriguing possibilities for potential cross-talk or cooperation between APP and calsyntenin-3.

The co-culture and neuron overexpression results shown here indicate that calsyntenin-3 can influence both excitatory and inhibitory synaptogenesis in culture.

Further examination of calsyntenin-3 localization may help determine whether this is also the case *in vivo*. In comparison, neuroligin-2 increases excitatory and inhibitory synaptogenesis in culture experiments; however, it is localized mainly at inhibitory synapses in the brain and thus has a much greater effect on inhibitory synapse function *in vivo* (Chih et al., 2005; Chubykin et al., 2007; Levinson et al., 2005; Varoqueaux et al., 2004). However, neuroligin-2 can be re-directed to excitatory synapses with overexpression of PSD-95 (Levinson et al., 2005). Initial reports, however, suggest that calsyntenin-3 is present at both excitatory and inhibitory postsynaptic compartments (Hintsch et al., 2002), so it may in fact exert synaptogenic effects at both types of synapses *in vivo*.

Ideally, the role of calsyntenin-3 in presynaptic induction should be confirmed by a complimentary knockdown approach using shRNA or RNAi. However, western blot experiments to assess the ratio of cleaved to full length calsyntenin-3 in our hippocampal culture system revealed that ~90% of total calsyntenin-3 is in the cleaved form (data not shown). Although we are limited by the lack of a good anti-calsyntenin-3 antibody for immunostaining to confirm these findings, the western blot experiments suggest that the endogeneous amount of full length calsyntenin-3 is very low in the hippocampal culture system. This high rate of cleavage also made it very difficult to assess overexpression effects in transfected neurons, as even transfected full length Myc-C3-CFP failed to accumulate on dendrite surfaces; the rate of cleavage of Myc-C3ΔPCS-CFP is presumably less as it can accumulate on dendrite surfaces at a high enough level to be detected. Due to the high rate of proteolytic processing in culture, loss of function experiments will ultimately have to

be done *in vivo* to determine the possible effects of a calsyntenin-3 deficiency at synapses.

The increased presynaptic clustering at excitatory and inhibitory synapses induced by calsyntenin-3 overexpression was accompanied by a dispersal of postsynaptic proteins. By lowering the expression level of calsyntenin-3, it is possible that postsynaptic protein clusters would be detectable. In the case of neuroligins, both decreases and increases in postsynaptic protein clustering have been reported in overexpression experiments (Chih et al., 2005; Graf et al., 2004; Prange et al., 2004); these discrepancies are likely due to differences in expression level and experimental protocols. Dispersal of postsynaptic protein clusters may occur via an indirect mechanism, by which the large increase in presynaptic protein clusters redistributes postsynaptic proteins, which may only be available in a limited supply. These postsynaptic clusters may become so spread out that puncta cannot be detected by immunostaining. Alternatively, dispersal of postsynaptic proteins may occur via a more direct mechanism, by which calsyntenin-3 actively recruits postsynaptic proteins via its intracellular domain to sites where it is being expressed. In the case of overexpression reported here, calsyntenin-3 filled the entire dendrite, so if postsynaptic proteins were recruited equally to calsyntenin-3 molecules, it is likely that puncta would no longer be present. Direct aggregation of tagged full length calsyntenin-3 as well as calsyntenin-3 lacking the intracellular domain on dendrite surfaces may help distinguish between these two possibilities, and would definitively show that calsyntenin-3 is able to mediate bi-directional synaptic signaling via intracellular interactions.

In the case of neuroligin-2, which also disperses postsynaptic proteins, the intracellular PDZ-binding domains are known to mediate many of the intracellular postsynaptic interactions that result in the clustering of scaffolding proteins and neurotransmitter receptors (Chubykin, 2009; Craig and Kang, 2007; Sudhof, 2008). For calsyntenins, the potential the intracellular binding partners are largely unknown, except for the reported binding to Mint2/XllL adaptor protein. However, other intracellular binding partners may also exist.

The binding assay presented here shows that calsyntenin-3 binds to neurexin-1α at high affinity. Neurexin-1α is expressed throughout the brain, including both pyramidal cells and interneurons in the hippocampus, so it is well-placed to potentially mediate both excitatory and inhibitory synaptogenesis via trans-synaptic signaling with calsyntenin-3 (Chubykin, 2009; Ullrich et al., 1995). Domain analysis also showed that both the cadherin and LNS domains of calsyntenin-3 were essential for presynaptic induction in co-culture. It will be interesting to determine if it is one or both of these domains that mediate binding to neurexin-1α. If one of these domains is not needed for neurexin binding, it is possible that they bind some other ligand to mediate synaptic development. The fact that the LNS DN/AA point mutation significantly decreased synaptogenesis in co-culture suggests that calcium binding may be involved. Neurexin binding to neuroligin is calcium-dependent, and this may also be the case for calsyntenin-3 – neurexin binding.

Interestingly, preliminary follow-up studies from the Craig lab show that calsyntenin-3 binding to neurexin is isoform-specific, binding only to α-neurexins but not β-neurexins. In this study, neurexin-1α(-SS4) was used for the binding assays,

but other preliminary results suggest that splice site 4 does not affect calsyntenin-3 binding. Thus, it appears that calsyntenin-3 may bind only α-neurexins(±SS4) but not β-neurexins. These findings support the emerging idea that neurexins may act as master organizers of synapses via isoform- and splice site-specific binding to a variety of postsynaptic ligands. In comparison, LRRTM-1 and -2 bind both α- and β-neurexins(-SS4) but not (+SS4), and the Cbln1-GluRδ2 complex binds α- and β-neurexins(+SS4) but not (-SS4) (Joo et al., 2011; Siddiqui and Craig, 2011). For neuroligins, neuroligin-1 with splice site B binds to β-neurexins but not α-neurexins, while neuroligins-1,-2,-3 and -4 without SSB can bind either α- or β-neurexins, with varying affinities depending on neurexin SS4 (Chubykin, 2009; Siddiqui and Craig, 2011). The calsyntenin-3 binding code appears to be distinct from these other families of postsynaptic molecules, suggesting it may have a unique function at synapses.

In conclusion, we identify calsyntenin-3 as a new synaptogenic protein, able to increase presynaptic protein clustering at both excitatory and inhibitory synapses via its extracellular domain. Although an ever-growing number of proteins have synapse-promoting abilities, calsyntenin-3 is fairly unique in that it is cleaved to release the extracellular domain. This provides intriguing possibilities for further study of the regulation of binding between synaptic CAMs: cleaved calsyntenin-3 may, via binding to neurexin, block synaptogenic signaling not only between membrane-anchored calsyntenin-3 and neurexin, but also between other neurexin ligands such as neuroligin and LRRTMs, and thus may have broader functions at synapses.

Chapter 3: MDGAs

3.1 Introduction

Autism spectrum disorders (ASDs) are common neurodevelopmental disorders characterized by impaired communication and language skills, social interaction deficits and stereotyped behavioural abnormalities. Onset of these disorders typically occurs before the age of three, during the time of peak synapse formation and maturation in humans (Huttenlocher and Dabholkar, 1997). The high co-incidence of ASDs with epilepsy (about 30%) has prompted speculation that the balance between excitatory and inhibitory neurotransmission (E/I) may be disrupted (Canitano, 2007). Imbalances in E/I ratios have also been suggested to be involved in schizophrenia and other neurological disorders. Interestingly, recent genetic studies in humans have implicated a number of synaptic cell adhesion molecules (CAMs) in both ASDs and schizophrenia (Peca et al., 2011b).

Two of these CAMs are neurexin and neuroligin, which are perhaps the best characterized synaptic partners (Chubykin, 2009; Craig and Kang, 2007). Neuroligins gained significant interest when they were reported as proteins able to induce presynaptic specializations in contacting axons (Scheiffele et al., 2000). Shortly thereafter, neurexins, the presynaptic binding partners for neuroligins, were reported to induce postsynaptic differentiation (Graf et al., 2004). Different isoforms of neurexins and neuroligins play specific roles in the formation of glutamatergic and GABAergic synapses, with neuroligin-1 being the main glutamatergic neuroligin, and neuroligin-2 localizing specifically to GABAergic synapses (Song et al., 1999; Varoqueaux et al., 2004). Furthermore, both neurexins and neuroligins are

alternatively spliced, and evidence suggests that splicing further influences synaptic specificity and binding affinities (Craig and Kang, 2007). Neuroligins clearly play an important role in synapse development, as a neuroligin-1, -2, -3 triple knockout (KO) is perinatally lethal due to synaptic transmission defects (Varoqueaux et al., 2006). Individual neuroligin-1 and -2 knockouts are viable, but have selective defects in glutamatergic or GABAergic development, respectively (Chubykin et al., 2007; Jedlicka et al., 2011; Poulopoulos et al., 2009). However, given that even triple neuroligin KO mice still form synapses, there are clearly other molecular mechanisms that modulate synaptic development and maintenance (Akins and Biederer, 2006; Dalva et al., 2007; Siddiqui and Craig, 2011). Neurexins have other binding partners at the synapse, including dystroglycans (Sugita et al., 2001), neurexophilins (Petrenko et al., 1996), LRRTM1 and 2 (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010), and the Clbn1-GluRδ2 complex (Uemura et al., 2010). However, alternate synaptic partners have not yet been reported for neuroligins.

Another family of proteins recently linked to neurodevelopmental disorders are MDGAs (MAM domain containing glycosylphosphatidylinositol anchor). Two independent genotyping studies in humans have associated intronic single nucleotide polymorphisms (SNPs) in MDGA1 to schizophrenia and bipolar disorder, (Kahler et al., 2008; Li et al., 2011), and another study found multiple copy number variants (CNVs) resulting in protein truncations in MDGA2 in autism (Bucan et al., 2009). MDGA1 was originally discovered in a differential PCR screen of rat basilar pons to identify secreted and transmembrane proteins that might be involved in neuronal migration, axon outgrowth and axon-target recognition (Litwack et al.,

2004). Sequence analysis and biochemical characterization revealed a monomeric protein encoding six immunoglobulin (Ig)-like domains, a fibronectin type III-like domain (FNIII) and an MAM (meprin, A5 protein, receptor protein tyrosine phosphatase mu) domain, as well as a glycosylphosphatidylinositol (GPI)-anchoring site (Litwack et al., 2004). Homology searches revealed a second family member, MDGA2, which has very similar domain structure to MDGA1, but lacks homology to the FNIII domain (Litwack et al., 2004).

Previous work shows that MDGA1 is expressed throughout the developing and mature nervous system, including the basilar pons, hippocampus, amygdala, olfactory bulb, cerebellum, thalamus, superficial cortical layers, and spinal cord (Lein et al., 2007; Litwack et al., 2004). MDGA2 is also highly expressed in the basilar pons but shows much lower expression than MDGA1 in most brain regions, including the hippocampus (Lein et al., 2007; Litwack et al., 2004). At the cellular level, MDGA1 is found on the cell surface, and mainly localizes to lipid rafts (Diaz-Lopez et al., 2005). MDGA1's expression in specific cortical layers during development suggests it may play a role in laminar or area patterning in the cortex (Takeuchi et al., 2007b), and RNAi against MDGA1 during this time results in defects in migration of layer 2/3 neurons (Takeuchi and O'Leary, 2006). A recent study with MDGA1-deficient mice showed no gross anatomical differences by postnatal day 14 (P14) (Ishikawa et al., 2011). However, closer inspection showed that cortical migration was delayed throughout the embryonic stages in a subset of cortical neurons, but these MDGA1-mutant neurons had largely reached their correct positions in the upper cortical layer by P7 (Ishikawa et al., 2011). Despite previous

work, it is still unclear which domains play a role in the functions of MDGAs, and any extracellular binding partners have not yet been reported.

Given the temporal and spatial expression patterns of MDGAs, the common cell adhesion domains in the MDGA1 and 2 protein structures, and the recent links to autism and schizophrenia, we hypothesized MDGAs may play a role in synaptic development. In this study, we report MDGA1 and 2 as new binding partners for neuroligin-2. Through induction, overexpression and function-blocking experiments, we show MDGA1 is a negative regulator of neuroligin-2, supporting the conclusion that MDGA1 specifically modulates inhibitory synaptic development through neuroligin-2.

3.2 Experimental Procedures

3.2.1 DNA Constructs

Full length rat MDGA1 and MDGA2 were amplified from a rat cDNA library (Linhoff et al., 2009) and subcloned into the spHA-C1 vector, which expresses the hemagglutinin (HA) tag with an N-terminal sequence directed from TrkC under a cytomegalovirus (CMV) promoter (Takahashi et al., 2011). The following deletion constructs for HA-MDGA1 were made by inverse PCR method: ΔIg1 (amino acids (aa) 24-123 deleted), ΔIg2 (aa 132-230 deleted), ΔIg3 (aa 240-323 deleted), ΔIg1-3 (aa 24-323 deleted), ΔIg4-6 (aa 338-632 deleted), ΔMAM (aa 752-919 deleted), ΔFNIII (aa 641-740 deleted), and Ig1-3 only (aa 338-919 deleted). YFP-MDGA1 was subcloned from HA-MDGA1 into the spYFP-C1 vector (Linhoff et al., 2009). HA-MDGA1 and Ig1-3 only were both subcloned into a vector with the CAG chicken β-

actin promoter for use in overexpression studies (kind gift from Dr. Gary Banker, Oregon Health Sciences University, Portland, OR) (Kaech and Banker, 2006; Niwa et al., 1991). HA-CD4 was subcloned from YFP-CD4 into the spHA-CD1 vector (Takahashi et al., 2011) to produce CD4 with an extracellular HA-tag. CMV-HA-neurexin1β(-SS4) was subcloned from HA-neurexin1β(-SS4)-pcGlobin2 (Gauthier et al., 2011), into spHA-C1, a vector that expresses HA with an N-terminal signal sequence derived from TrkC cDNA (Takahashi et al., 2011). Previously described plasmids include CFP-neuroligin 2 (CFP-Nlg2), CFP-neuroligin 1 (CFP-Nlg1) and neurexin 1β(+SS4) fused to Fc (Nrx1β(+SS4)-Fc) (Graf et al., 2006; Graf et al., 2004). Nlg1-Fc and Nlg2-Fc were subcloned from Nlg1-CFP and Nlg2-CFP, respectively into pc4-sp-Fc2, pcDNA4 with the following sequences inserted between HindIII and XhoI: neurexin1beta signal sequence followed by a multiple cloning site (EcoRV-NheI-EcoRI-BamHI-NotI) and next the human IgG Fc cDNA containing stop codon.

For plasmid-based RNA inhibition of MDGA1, the complementary oligonucleotide encoding inverted repeats that target nucleotides 1027-1045 of rat MDGA1 (GCATCCCTGACAAGTCTAT) for sh-MDGA1 was annealed. The construct for expressing MDGA1 resistant against sh-MDGA1 (MDGA1*) was generated by making the following six point mutations, indicated by underlines, in the shRNA-targeting site: GCATACCGGATAAAAGTAT for sh-MDGA1 resistance. As a control shRNA, we used shMORB (here called sh-con) that mediates knockdown of MORF4L1 involved in chromatin regulation but has no effects on neuronal

morphology including spine density (Alvarez et al., 2006), and does not induce any interferon response (Bridge et al., 2003).

3.2.2 Cell Culture and Transfection

Dissociated primary hippocampal neuron cultures were prepared from embryonic day 18 rat embryos as described previously (Banker and Goslin, 1998; Kaech and Banker, 2006). Neurons were plated at a density of 300,000 cells per dish on poly-L-lysine coated coverslips and inverted over a feeder layer of glia in 60mm culture dishes. To prevent overgrowth of glia, cytosine arabinoside (5 μM) was added to neuron cultures at 2 d. Serum-free media was also supplemented with 100 μM APV (Research Biochemicals) to prevent excitotoxicity. For overexpression and shRNA studies, neurons were transfected with 1-5 μg of DNA per dish at day *in vitro* (DIV) 8-9 using the ProFection Mammalian Transfection System (Promega). Neurons were fixed at 14 DIV. For localization studies, neurons were transfected with 3 μg of DNA using the AMAXA nucleofector system (Kit: VPG-1003, Program: O-003, Lonza) at 0 DIV, and fixed at either 16 DIV or 21 DIV.

COS7 and HEK293T cells were cultured in DMEM-H media with 10% fetal bovine serum. All transfections of COS7 cells for binding assays and co-cultures were performed using TransIT-LT1 Transfection Reagent (Mirus), using 0.125– 2 μg of DNA. Co-cultures of primary hippocampal neurons with COS7 cells were performed as described previously (Graf et al., 2004). Briefly, transfected COS7 cells were trypsinized and seeded onto neuron coverslips grown for 9-10 DIV, then fixed 20-24 h later.

3.2.3 Western Blotting

For validation of sh-MDGA1, HEK cells and primary cortical neurons were transfected as described above with various combinations of sh-MDGA1 or sh-con with HA-MDGA1, HA-MDGA1* or HA-MDGA2. After 24-48 hours of expression, the cells were washed with phosphate buffered saline then scraped into lysis buffer (1% triton X-100, 150mM NaCl, 20mM Tris pH 7.4, 1mM DTT, 1mM EDTA, plus protease inhibitor tablet) (Sigma, Roche). Lysates were centrifuged at 16000 x g for 15 min at 4°C and the supernatant was collected. The protein concentrations of lysates were determined using the Bio-Rad Protein Assay kit (Bio-Rad), using BSA as a standard (Sigma). Protein concentrations were normalized between samples and loaded into 10% polyacrylamide gels with an equal volume of loading buffer. Gels were run and transferred using the Hoeffer Mini Western Electrophoresis System with Transfer Tank (Hoeffer). Transfer was completed onto Immobilon P membranes (Millipore), blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 (Sigma) and incubated with primary (anti-HA mouse IgG2b; clone 12CA5; Roche), and secondary (Goat anti-mouse HRP conjugate; Millipore) antibodies. Immunoblots were detected using the SuperSignal Chemiluminescent kit (Thermo Scientific) and visualized by chemiluminescence using a Bio-Rad gel documentation system.

3.2.4 Production of Soluble Fc-fusion Proteins

Expression of Nlg2-Fc protein was performed by transfecting HEK293T cells with the encoding plasmid, and culturing in DMEM with 10% FBS and 0.5 mg/ml Zeocin (Invitrogen). After 21-day selection with Zeocin, medium was replaced with serum-free AIM V synthetic medium (Invitrogen). The conditioned medium was

collected every 2-3 days for three weeks and frozen at -80°C, for a total of 300-400 mL. Expression of the human Fc tagged fusion proteins Nlg1-Fc and Nrxn1β(+S4)-Fc was performed by transient transfection with the encoding plasmids in HEK293T cells. A day after transfection, DMEM containing 10% FBS was replaced with serum-free AIM V synthetic medium. After 48h, approximately 120 mL of conditioned medium was collected. Medium was purified using protein-G sepharose 4 fastflow columns (GE Healthcare) and concentrated in PBS with Centricon filters (Millipore). Purified Fc fusion proteins were immunoblotted, visualized by chemiluminescence using a Bio-Rad gel documentation system, and quantitated by densitometry relative to a human IgG standard curve.

3.2.5 Binding Assays

To assess binding between MDGA1 and Nlg1 / Nlg2, COS7 cells growing on coverslips were transfected with MDGA1 wild type or deletion constructs, neurexin1β or negative control CD4 and allowed to express for 24 h. Cells were incubated with fusion proteins (Nlg1-Fc or Nlg2-Fc) live for 1 h at 20°C, followed by anti-HA antibodies (1:500; IgG2b; clone 12CA5; Roche) for 30 min. Binding was assayed in the following “binding buffer”: 168 mM NaCl, 2.6 mM KCl, 10 mM HEPES, pH 7.2, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, and 100 µg/ml BSA. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4)) for 15 min at 20°C then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C. This was followed by incubation with secondary antibodies FITC-conjugated donkey anti-human IgG (H+L) (1:100, Jackson ImmunoResearch) and Alexa-568 anti-IgG2b (1:1000, Invitrogen) for 1 h at 37°C to

visualize bound-Fc protein and surface HA, respectively. Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

Similarly, to assess binding of neurexin1β, COS7 cells growing on coverslips were transfected with CFP-Nlg2 and HA-MDGA1 or HA-CD4 and allowed to express for 24 h. Cells were incubated with Nrxn1β-Fc fusion protein live for 1 h at 20°C, followed by anti-HA antibodies (1:500; IgG2b; clone 12CA5; Roche) for 30 min. Cells were then fixed in parafix and stained with secondary antibodies in the same way described above.

3.2.6 Immunocytochemistry

For staining neuron cultures or neuron-COS7 co-cultures, the following protocol was used. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4) for 15 min followed by permeabilization with PBST (PBS + 0.25 % Triton X-100) or in -20°C methanol for 10 min. They were then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C, followed by incubation with primary antibodies in blocking solution (overnight, 20°C) and secondary antibodies (45 min, 37°C). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

For surface labeling of HA- or YFP- signals in neurons, the same protocol was followed except that cells were incubated with anti-HA (1:500; IgG2b; clone 12CA5; Roche) or anti-GFP (1:500; rabbit; A11122; Invitrogen) antibodies for 1 h at 37°C following fixation in parafix solution, but prior to permeabilization with PBST.

For determining surface expression in COS7 cells, anti-GFP antibody in "binding buffer" was incubated with cells for 30 min at 20°C. Cells were then fixed in parafix solution, permeabilized, blocked and incubated with primary and secondary antibodies as described above.

The following polyclonal primary antibodies were used: rabbit anti-synapsin I (1:2000; Millipore; AB1543P), rabbit anti-VGlut1 (1:2000; Synaptic Systems; 135 302), rabbit anti-VGAT (1:1000; Synaptic Systems; 131 003), rabbit anti-Neuroligin-2 (1:500; rabbit polyclonal; Zymed, as in (Graf et al., 2006)). The following mouse monoclonal antibodies were used: anti-PSD-95 family (1:500; IgG2a; clone 6G6-1C9; Thermo Scientific; recognizes PSD-95, PSD-93, SAP102 and SAP97), anti-gephyrin (1:500; IgG1; mAb7a; Synaptic Systems), anti-HA (1:1000; IgG2b; clone 12CA5; Roche). For labeling dendrites, we used anti-MAP2 (1:4000, chicken polyclonal IgY; Abcam; ab5392).

Secondary antibodies were raised in goat against the appropriate species and monoclonal isotype, highly cross-absorbed and conjugated to Alexa-488, Alexa-568 and Alexa-647 dyes (1:500, Invitrogen). AMCA (7-amino-4methylcoumarin-3-acetic acid)-conjugated anti-chicken IgY (donkey IgG; 1:200; Jackson ImmunoResearch; 703-155-155) was used for visualizing dendrites.

3.2.7 Imaging, Image Analysis and Statistical Analysis

Images were acquired on a Zeiss Axioplan2 microscope with a 40X 1.30 NA oil objective, a 63X 1.4 NA oil objective or a 25X 0.8 NA oil objective and Photometrics Sensys cooled CCD camera using Metamorph imaging software (Molecular Devices) and customized filter sets. Images were initially acquired as 12-

bit grayscale and were prepared for presentation using Adobe Photoshop (Adobe Systems). For quantification, sets of cells were fixed and stained simultaneously and imaged with identical settings. All image acquisition, analysis and quantification were done blind to the experimental condition.

To determine the binding affinity of soluble proteins to surface-expressed proteins, regions were drawn around the perimeter of each COS7 cell, and the average intensity values of bound protein and expressed protein were measured within the region. Average off-cell background measures were subtracted from these values to yield corrected average intensity values for bound protein and expressed protein. Similar methods were employed to determine surface expression compared to total expression of CFP-tagged proteins in COS7 cells.

For co-cultures, two methods of analysis were used. For co-cultures comparing w.t. HA-MDGA1 + CFP-Nlg1 or -Nlg2, fields for imaging were chosen based on CFP, HA, MAP2 and phase channels, for the presence of COS7 cells expressing both MDGA1 and Nlg1/2 in a neurite-rich area. During analysis, regions were created around the expressing COS7 cells that excluded MAP2-positive areas so as to exclude endogeneous synapses, and the total grey value of all puncta in the synapsin channel was thresholded and measured. The total area measurements from the HA-channel were used to normalize measures to COS7 cell area.

For the co-cultures comparing various MDGA deletion constructs, cells were chosen based on CFP, HA, MAP2 and phase channels, for the presence of COS7 cells expressing both MDGA1 and Nlg1/2 in a neurite-rich area. Cells were then

scored as positive or negative for synapsin clustering over the expressing COS7 cell without MAP2 signal.

For analysis of neurons in the overexpression and shRNA experiments, neurons were chosen for imaging based on HA-signal (for overexpression) or CFP-signal (for shRNA), as well as healthy morphology under phase contrast and MAP2 channels. Neighbouring cells for overexpression analysis were chosen based on similar MAP2 staining. During analysis, regions were created around single expressing or non-expressing dendrites and thresholded in the VGAT or VGlut1 and gephyrin or PSD95 channels. Total number of puncta was measured, as well as puncta with overlapping pixels between the pre- and post-synaptic channels. Measures were corrected for off-cell background and normalized to dendrite length.

Analysis was performed using Metamorph (Molecular Devices), Excel (Microsoft) and GraphPad Prism (GraphPad Software). Statistical comparisons were made with Student's unpaired t-test or one-way ANOVA with post hoc Bonferroni's multiple comparison test, as indicated in figure legends. All data are reported as the mean ± standard error of the mean (SEM).

3.3 Results

3.3.1 MDGA1 and MDGA2 Bind Neuroligin-2

Based on the brain-specific expression of MDGAs (Litwack et al., 2004), the presence of cell adhesion domains, and links to ASD and schizophrenia (Bucan et al., 2009; Kahler et al., 2008; Li et al., 2011), we hypothesized that MDGAs may play a role in synaptic development. Initial tests in neuron-fibroblast co-culture hemi-

synapse induction assays, as detailed further below, led us to suspect that MDGAs might interact with neuroligins. Since MDGAs are GPI-anchored, such an interaction would have to occur via extracellular domains. A common method to demonstrate interaction of protein extracellular domains such as for neurexin-neuroligin is to incubate cultured cells expressing neurexin with soluble recombinant neuroligin ectodomain or vice-versa (Boucard et al., 2005; Chih et al., 2006; Siddiqui et al., 2010). To characterize the binding between MDGA1 or MDGA2 and neuroligins, we generated soluble ectodomain fusion proteins of neuroligins with the Fc (Fragment, crystallizable) antibody region (Nlg1-Fc and Nlg2-Fc). We assayed binding of these Fc proteins to COS cells expressing MDGAs. Binding of Nlg2-Fc was observed for both MDGA1 and MDGA2, similar to that observed for neurexin1β, whereas no binding was observed to nontransfected cell or cells expressing negative control (lymphocyte protein CD4) (Figure 3.1, a). To further characterize this interaction, we performed binding assays over a range of concentrations of Nlg2-Fc. We observed saturated binding for both HA-MDGA1 and HA-MDGA2 (Figure 3.1, b). By Scatchard analysis, we found an apparent dissociation constant (Kd) of 7.3 ± 1.0 nM for Nlg2-Fc binding to MDGA1 and 45.9 ± 11.9 nM for Nlg2-Fc binding to MDGA2. In the same assays, the Kd for neurexin1β binding to Nlg2-Fc was 8.4 ± 1.1 nM. These binding affinities are in the nanomolar range typically observed in ligand-receptor interactions. Based on the lower binding affinity of MDGA2, as well as the much lower expression in the brain (Lein et al., 2007), we focused on MDGA1 for the remainder of our analysis.

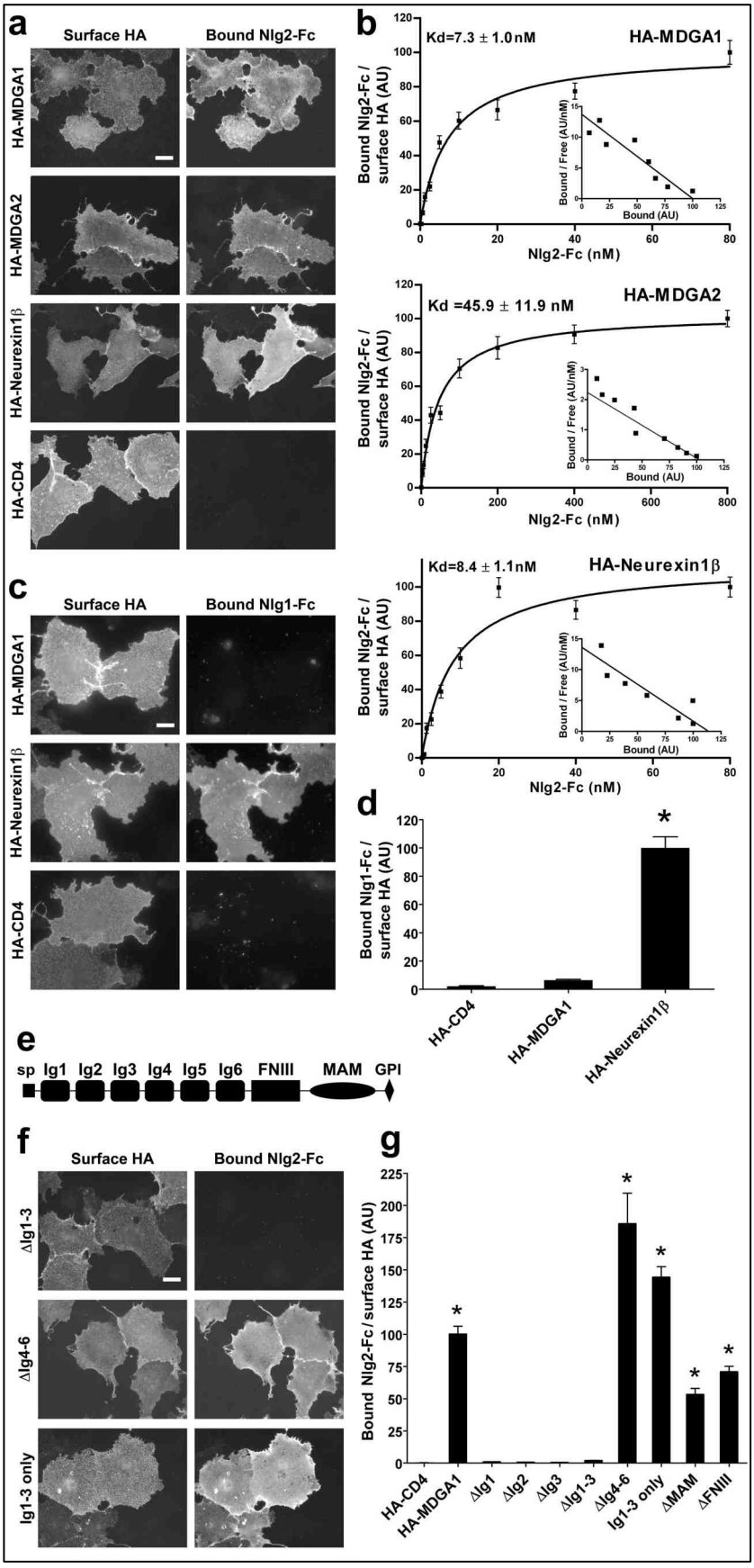
a
Surface HA
Bound Nlg2-Fc
HA-MDGA1
HA-MDGA2
HA-Neurexin1β
HA-CD4
b
Kd=7.3 ± 1.0nM
HA-MDGA1
Kd =45.9 ± 11.9 nM
HA-MDGA2
Kd=8.4 ± 1.1nM
HA-Neurexin1β
Bound Nlg2-Fc / surface HA (AU)
Nlg2-Fc (nM)
Bound / Free (AU/nM)
Bound (AU)
c
Surface HA
Bound Nlg1-Fc
d
Bound Nlg1-Fc / surface HA (AU)
e
sp Ig1 Ig2 Ig3 Ig4 Ig5 Ig6 FNIII MAM GPI
f
Surface HA
Bound Nlg2-Fc
ΔIg1-3
ΔIg4-6
Ig1-3 only
g
Bound Nlg2-Fc / surface HA (AU)
HA-CD4
HA-MDGA1
ΔIg1
ΔIg2
ΔIg3
ΔIg1-3
ΔIg4-6
Ig1-3 only
ΔMAM
ΔFNIII

Figure 3.1: MDGA1 Binds with High Affinity to Neuroligin-2, but not Neuroligin-1. MDGA2 Also Binds Neuroligin-2
(a) Binding assay of Neuroligin-2-Fc protein (Nlg2-Fc) to COS cells expressing HA-tagged constructs. Nlg2-Fc specifically bound to COS cells expressing HA-MDGA1 (top), HA-MDGA2 (second row) or HA-Neurexin1β (third row), but not HA-CD4 (bottom row). (b) By Scatchard analysis, affinity of binding of Nlg2-Fc to HA-MDGA1 (top curve), HA-MDGA2 (middle curve) and HA-Neurexin1β (bottom curve) was estimated at 7.3 nM, 45.9 nM and 8.4 nM, respectively (n = 20 cells each data point). (c) Binding assay of Neuroligin-1-Fc protein (Nlg1-Fc) to COS cells expressing HA-tagged constructs. Nlg1-Fc specifically bound to COS cells expressing HA-Neurexin1β (middle row), but not HA-MDGA1 (top row) or HA-CD4 (bottom row). (d) Quantitation of Nlg1-Fc bound to COS cells expressing the indicated HA-tagged constructs, normalized to HA-neurexin1β, assayed by incubating expressing cells with 200 nM Nlg1-Fc. ANOVA, < 0.0001, n = 30 cells each; *P < 0.001 for HA-Neurexin1β compared to HA-CD4 by post-hoc Bonferroni test. (e) Structure of MDGA1. MDGA1 has a signal peptide (sp), six immunoglobulin (Ig) repeats, followed by a fibronectin type 3 domain (FNIII) and a meprin, A5 protein, receptor tyrosine phosphatase mu (MAM) domain. It is anchored via a GPI-link to the plasma membrane at the C-terminus. (f) Representative images from binding assay of Nlg2-Fc to COS cells expressing MDGA1 deletion constructs. COS cells expressing ΔIg1-3 did not bind Nlg2-Fc (top row), but cells expressing ΔIg4-6 (middle row) or Ig1-3only (bottom row) did bind Nlg2-Fc. (g) Quantitation of Nlg2-Fc binding to MDGA1 deletion constructs in COS cells, normalized to HA-MDGA1. The first three Ig repeats (Ig1-3) are necessary and sufficient for binding Nlg2-Fc. ANOVA, P < 0.0001, n ≥ 20 cells each in two independent experiments; *P < 0.001 compared to HA-CD4 by post-hoc Bonferroni test. All results are expressed as mean ± SEM. Scale bars are 20 μm.

We next tested binding of neuroligin-1(+SSB) to MDGAs in the same way. Although there appeared to be some signal associated with HA-MDGA1-expressing cells when incubated with very high concentrations of Nlg1-Fc protein, we were unable to observe saturated binding (not shown). At 200 nM of Nlg1-Fc protein, MDGA1 binding was not significantly higher than a non-specific negative control CD4 (Figure 3.1, c and d). We could observe no binding of Nlg1-Fc to cells expressing HA-MDGA2 (not shown). Thus, if MDGAs interact with neuroligin-1(+SSB), it must be a low affinity interaction, considerably weaker than that of MDGAs with neuroligin-2.

MDGA1 has six Ig-like repeats (Ig), a fibronectin type III domain (FNIII), and an MAM domain (Figure 3.1, e). To determine the domains responsible for binding to neuroligin-2, various deletion constructs were cloned to eliminate each of the first three Ig repeats individually (ΔIg1, ΔIg2, ΔIg3), the first three Ig repeats together

(ΔIg1-3), the last three Ig repeats together (ΔIg4-6), the FNIII domain (ΔFNIII), the MAM domain (ΔMAM), and all domains except the first three Ig domains (Ig1-3 only). These constructs were transfected into COS cells and assayed for binding to Nlg2-Fc (Figure 3.1, f and g). Binding was abolished when any one of the first three Ig repeats were removed, while binding was preserved with only the first three Ig repeats present, indicating that the Ig repeats 1-3 are necessary and sufficient for MDGA1 binding to neuroligin-2. The Ig 4-6 repeats, MAM or FNIII domains do not appear to play a role in binding to neuroligin-2 as binding was not significantly different than that observed for full length MDGA1 when they were deleted (Figure 3.1, g).

3.3.2 MDGA1 Partially Localizes at Synapses with Neuroligin-2

We next examined subcellular localization of MDGA1 in cultured hippocampal neurons. In the absence of any MDGA1 antibody suitable for immunofluorescence, we assessed localization of recombinant MDGA1 extracellularly-tagged with yellow fluorescent protein (YFP-MDGA1) expressed at low level in cultured neurons. At 14-21 days in vitro (DIV), YFP-MDGA1 was partially diffuse in dendrites and axons, and partially concentrated at punctate sites in dendrites (Figure 3.2, a). Some YFP-MDGA1 clusters co-localized with the inhibitory postsynaptic scaffold gephyrin and with neuroglin-2, suggesting partial localization to inhibitory sites (Figure 3.2, a and b). Most other YFP-MDGA1 clusters co-localized with PSD-95, suggesting partial localization to excitatory sites as well (Figure 3.2, c). Therefore, MDGA1 is well-positioned in hippocampal neurons to interact with neuroligin-2 at inhibitory synapses but can also target to excitatory synapses.

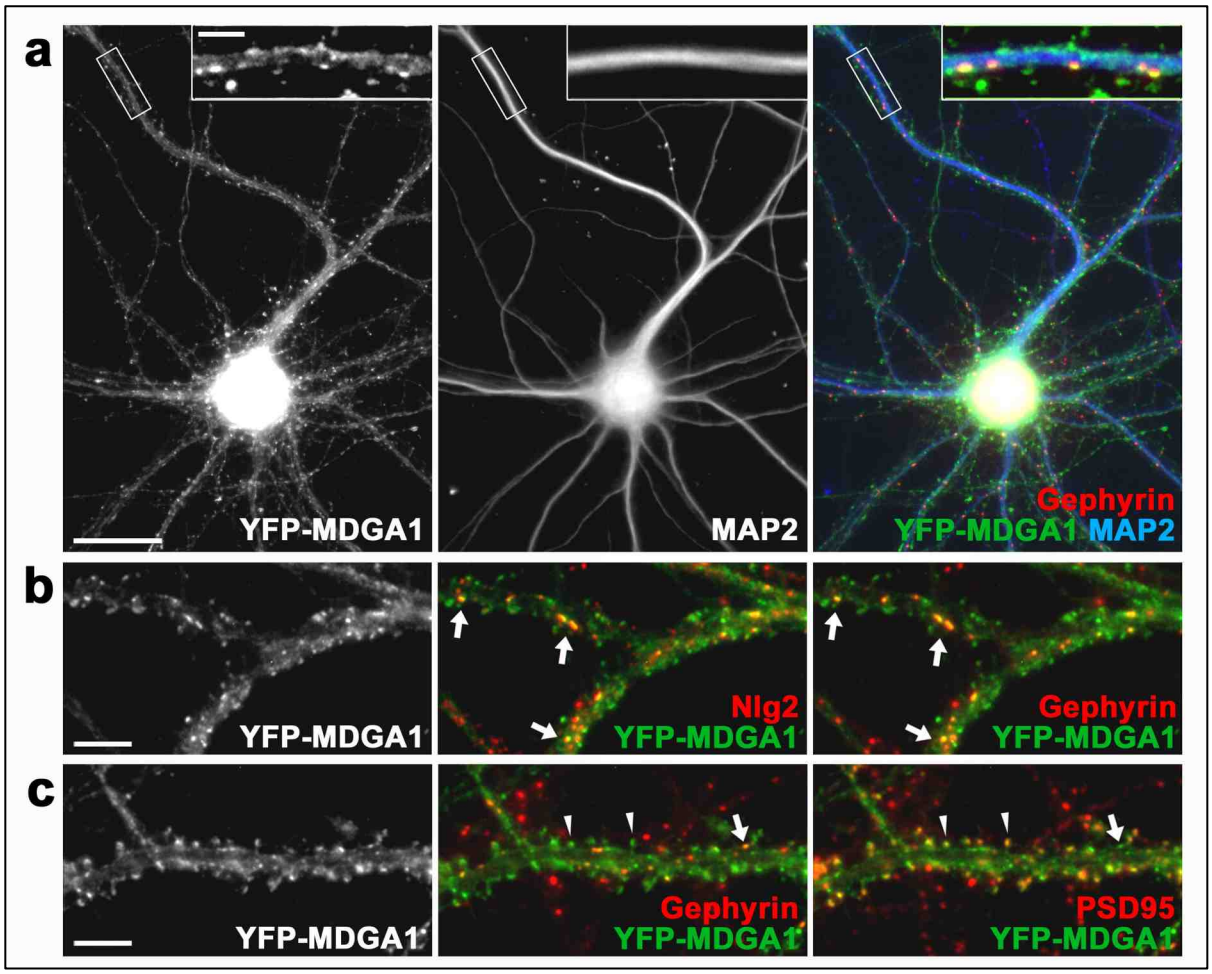

Figure 3.2: Recombinant MDGA1 Partially Co-localizes with Neuroligin-2 at Inhibitory Postsynaptic Sites

(a) Recombinant extracellularly tagged YFP-MDGA1 expressed at low level in cultured hippocampal neurons at 16 DIV is partially diffuse in dendrites and axons and partially concentrated at punctate sites in dendrites (left panel). Some MDGA1 clusters (green in inset of right panel) overlap with gephyrin clusters (red) on dendrites labeled with MAP2 (blue). (b) In cultured hippocampal neurons at 21 DIV, some YFP-MDGA1 clusters in dendrites overlap with neuroligin-2 (Nlg2) clusters (red in middle panel) as well as with gephyrin clusters (red in right panel). (c) In cultured hippocampal neurons at 21 DIV, a majority of the YFP-MDGA1 clusters not overlapping with gephyrin clusters overlap with PSD95 clusters (red in right panel). Arrow indicates YFP-MDGA1 clusters overlapping with gephyrin clusters, whereas arrowheads indicate YFP-MDGA1 clusters overlapping with PSD95 clusters. Representative images shown, n = three independent cultures. Scale bars represent 20 μm (a, left), 3 μm (inset in a) and 5 μm (b and c).

3.3.3 MDGA1 Inhibits Induction of Presynaptic Protein Clustering by Neuroligin-2

To further characterize the interaction between MDGA1 and neuroligin-2, we used the neuron-fibroblast co-culture assay (Graf et al., 2004). When expressed in COS cells, neuroligin-1 and -2 both induce clustering of presynaptic proteins such as synapsin (Scheiffele et al., 2000). In our assays, we co-transfected COS cells with MDGA1 and neuroligin-2. When COS cells were expressing both constructs simultaneously, the presynaptic protein clustering over transfected cells was greatly diminished, compared to cells expressing neuroligin-2 alone (Figure 3.3, a and d). Quantitatively, co-expression of HA-MDGA1 with CFP-neuroligin-2 significantly reduced synapsin clustering in axons contacting transfected COS cells compared with co-expression of control protein HA-CD4 (Figure 3.3, b and d). When we tried similar assays co-expressing MDGA1 with neuroligin-1, no reduction in presynaptic protein clustering was observed compared to co-transfection with CD4 (Figure 3.3, c and e). Thus, MDGA1 selectively inhibits the synaptogenic activity of neuroligin-2.

To determine which domains of MDGA1 are important for the inhibition of neuroligin-2 in co-culture, we carried out similar co-culture experiments co-expressing neuroligin-2 with various MDGA1 deletion constructs. As in our binding assays where the Ig1-3 repeats were necessary and sufficient for binding, we found that deleting Ig repeats 1-3 restored full presynaptic induction activity for neuroligin-2 (Figure 3.3, f and h), while co-expressing the Ig1-3 only construct was sufficient to suppress neuroligin-2 to the same extent as full length MDGA1 (Figure 3.3, g and h).

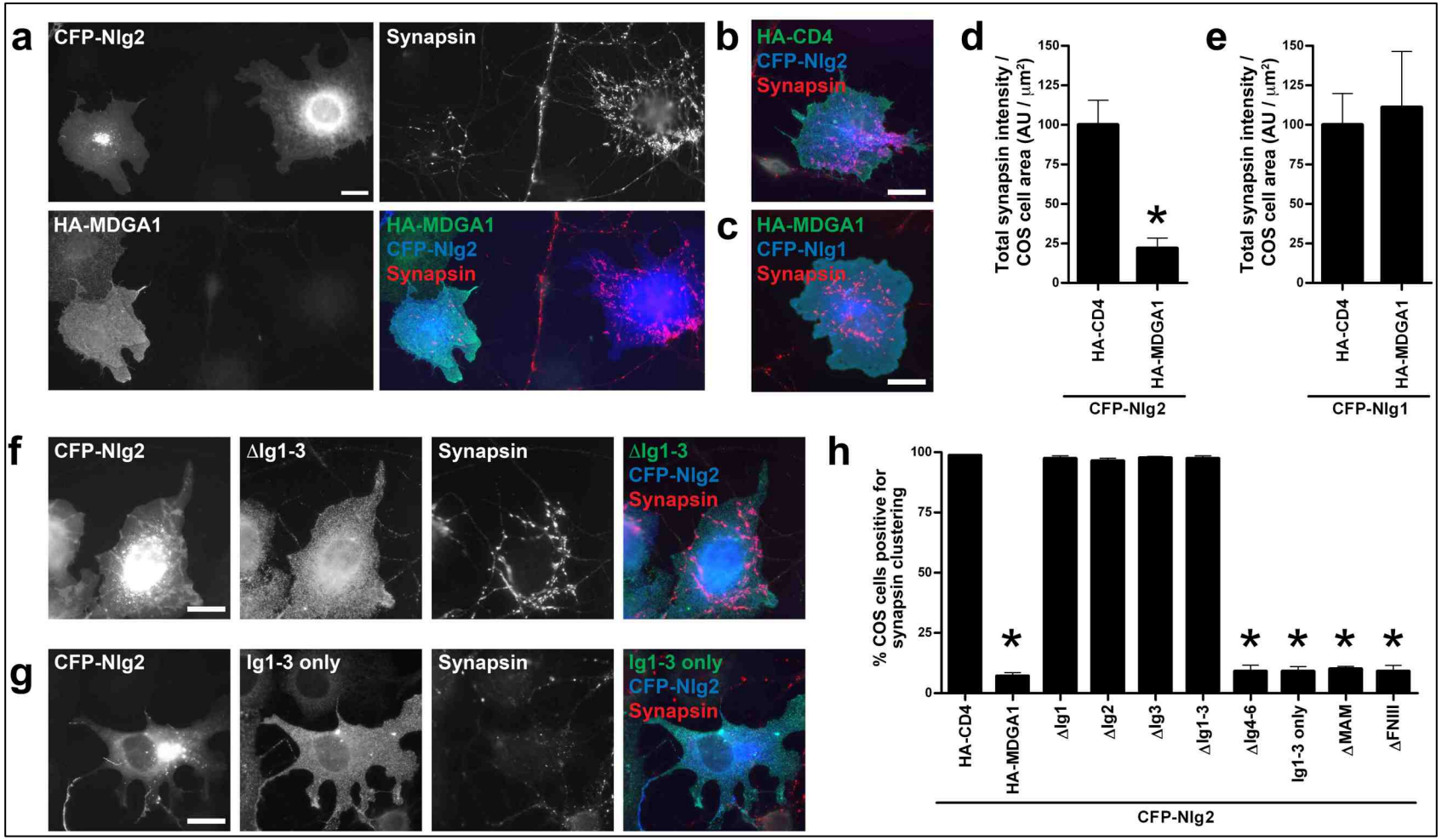
a
CFP-Nlg2
Synapsin
HA-MDGA1
HA-MDGA1
CFP-Nlg2
Synapsin
b
HA-CD4
CFP-Nlg2
Synapsin
c
HA-MDGA1
CFP-Nlg1
Synapsin
d
Total synapsin intensity / COS cell area (AU / μm²)
150
125
100
75
50
25
0
*
HA-CD4
HA-MDGA1
CFP-Nlg2
e
Total synapsin intensity / COS cell area (AU / μm²)
150
125
100
75
50
25
0
HA-CD4
HA-MDGA1
CFP-Nlg1
f
CFP-Nlg2
ΔIg1-3
Synapsin
ΔIg1-3
CFP-Nlg2
Synapsin
g
CFP-Nlg2
Ig1-3 only
Synapsin
Ig1-3 only
CFP-Nlg2
Synapsin
h
% COS cells positive for synapsin clustering
100
75
50
25
0
*
*
*
*
*
HA-CD4
HA-MDGA1
ΔIg1
ΔIg2
ΔIg3
ΔIg1-3
ΔIg4-6
Ig1-3 only
ΔMAM
ΔFNIII
CFP-Nlg2

Figure 3.3: MDGA1 Inhibits Presynaptic Induction by Neuroligin-2 in Co-culture
(a) COS cells were co-transfected with CFP-Nlg2 (blue) and HA-MDGA1 (green), and co-cultured with hippocampal neurons. Cells expressing only CFP-Nlg2 induce clustering of synapsin (red in bottom right panel) in contacting axons (COS cell on right), while clustering is greatly decreased in cells expressing both CFP-Nlg2 and HA-MDGA1 (COS cell on left). (b) Co-expression of HA-CD4 (green) with CFP-Nlg2 (blue) in COS cells did not result in a decrease in presynaptic induction of synapsin (red). (c) Co-expression of CFP-Nlg1 (blue) with HA-MDGA1 (green) did not decrease presynaptic induction of synapsin (red) by CFP-Nlg1. (d) Quantitation of the synapsin clustering associated with co-expressing COS cells and not with MAP2-postitive dendrites in a Nlg2 co-culture assay, normalized to HA-CD4 + CFP-Nlg2. T-test, $*P < 0.0001$, n = 10 cells each in two independent experiments. (e) Quantitation of the synapsin clustering associated with co-expressing COS cells and not with MAP2-postitive dendrites in a Nlg1 co-culture assay, normalized to HA-CD4 + CFP-Nlg1. T-test, $P = 0.7849$, n = 10 cells each in two independent experiments. (f, g) Representative images from Nlg2 co-cultures co-expressing various MDGA deletion constructs in COS cells. ΔIg1-3 does not decrease presynaptic induction by CFP-Nlg2, but Ig1-3 only decreases synapsin similar to full length MDGA1 when co-expressed with Nlg2 in co-culture. (h) Quantitation of the effect of various HA-tagged MDGA1 deletion constructs when co-expressed with Nlg2 in COS cells and co-cultured with neurons. The number of co-transfected cells positive for synapsin without MAP2 was counted and results are expressed as percentages. The Ig1-3 repeats are necessary and sufficient for the suppression of Nlg2-mediated synapsin clustering. ANOVA, $P < 0.0001$, n ≥ 3 experiments counting ≥ 100 cells per experiment; $*P < 0.01$ compared to HA-CD4 with CFP-Nlg2 by post-hoc Bonferroni test. All results are expressed as mean ± SEM. Scale bars are 20 μm.

The Ig 4-6 repeats, MAM domain and FNIII domains also had no effect on neuroligin-2 activity in co-culture (Figure 3.3, h). These results confirm that the Ig 1-3 domains, which are required for binding to neuroligin-2, are also essential for the inhibitory effect in co-culture.

3.3.4 MDGA1 Blocks Binding of Neuroligin-2 to Neurexin1β, but Does Not Affect Surface Trafficking of Neuroligin-2

Since neurexins are the binding partners of neuroligins that facilitate presynaptic induction in co-culture by neuroligins, we hypothesized that perhaps MDGA1 blocks this interaction. To test this hypothesis, we co-transfected COS cells with neuroligin-2 and MDGA1, and assayed binding to cells using a soluble neurexin fusion protein (Nrxn1β-Fc). In cell expressing only neuroligin-2, strong binding of neurexin1β was observed as expected, but in cells expressing both neuroligin-2 and

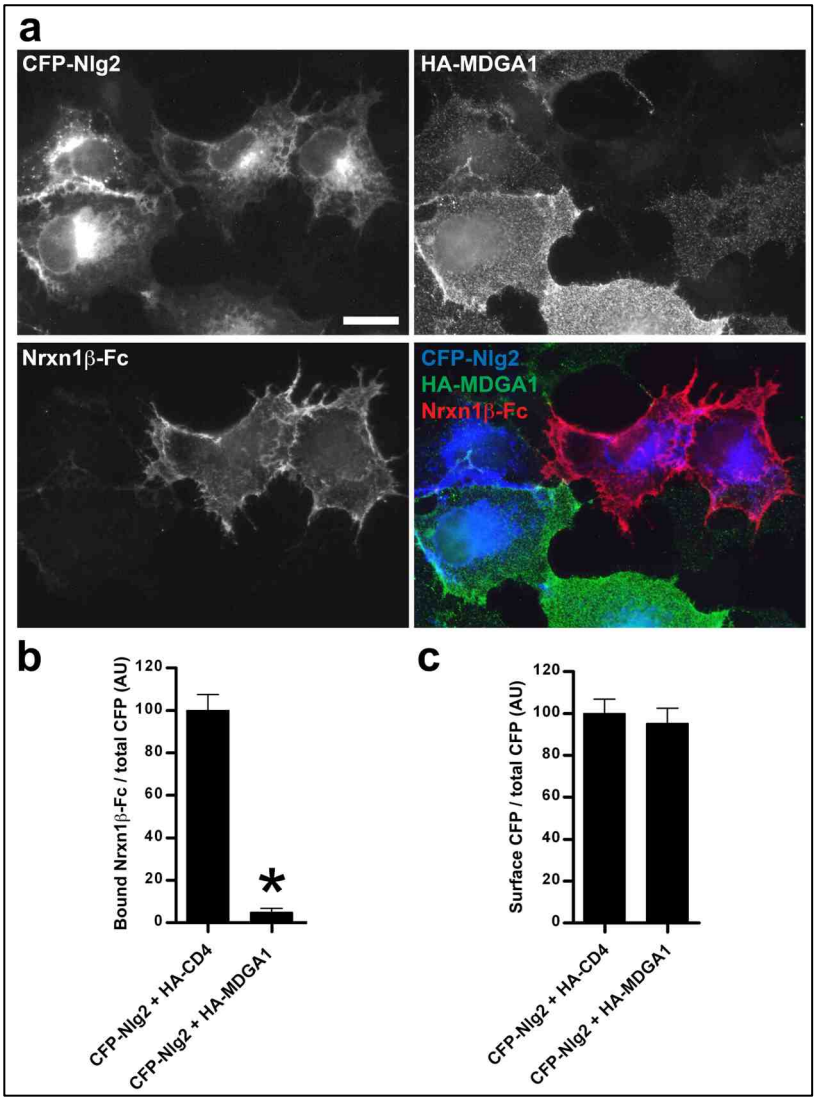

Figure 3.4: MDGA1 Blocks Neurexin1β Binding to Neuroligin-2, but Does Not Affect Surface Trafficking of Neuroligin-2

(a) COS cells were co-transfected with HA-MDGA1 (green) and CFP-Nlg2 (blue). Cells expressing only CFP-Nlg2 (cells in top right of image) bound soluble neurexin1β-Fc protein (Nrxn1β-Fc) (red), but cells expressing both CFP-Nlg2 and HA-MDGA1 (cells in bottom left of image) did not. Scale bar is 20 μm. (b) Quantitation of Nrxn1β-Fc binding in COS cells co-expressing Nlg2 and MDGA1, normalized to CFP-Nlg2 + HA-CD4. T-test, $P < 0.0001$, n = 10 cells each in two independent experiments. (c) COS cells co-expressing CFP-Nlg2 and HA-MDGA1 had no change in CFP-Nlg2 surface expression (measured using an anti-CFP antibody on live cells) compared to control co-transfection with HA-CD4. Data normalized to HA-CD4 + CFP-Nlg2. T-test, $P = 0.6317$, n = 10 cells each in two independent experiments. Results are expressed as mean ± SEM.

MDGA1, binding of neurexin fusion protein was completely abolished (Figure 3.4, a). Quantitatively, co-expression of MDGA1 significantly reduced binding of Nrxn1β-Fc to cells expressing neuroligin-2 compared with co-expression of control protein CD4 (Figure 3.4, b). However, when we analyzed cell surface levels of the CFP-tagged neuroligin-2, we found no significant difference in cells co-expressing neuroligin-2 and MDGA1 verses neuroligin-2 and a control CD4 construct (Figure 3.4, c). This data suggests that MDGA1 blocks binding of neuroligin-2 to its partner neurexin1β in some direct way on the cell surface, and does not interfere with surface trafficking of neuroligin-2.

3.3.5 Overexpression of MDGA1 Decreases Inhibitory Synapse Development in Culture

Next, we tested the effects of MDGA1 overexpression (DIV 9 – DIV 14) in cultured hippocampal neurons (Figure 3.5, a and b). Overexpression of MDGA1 significantly reduced the number of VGAT and gephyrin puncta, as well as inhibitory synapses (marked by gephyrin puncta apposed to VGAT puncta), compared to neighboring non-transfected neurons (Figure 3.5, c-e). There was no significant difference in inhibitory synapse markers when the ΔIg1-3 version was overexpressed, suggesting that binding to neuroligin is needed to mediate the decrease in inhibitory synapses. We also examined the number of excitatory synapses, but found no significant change when overexpressing either wild type or ΔIg1-3 MDGA1, compared to non-transfected neighbors (Figure 3.5, a, b and f). Thus, MDGA1 overexpressed in culture specifically reduces inhibitory synapses.

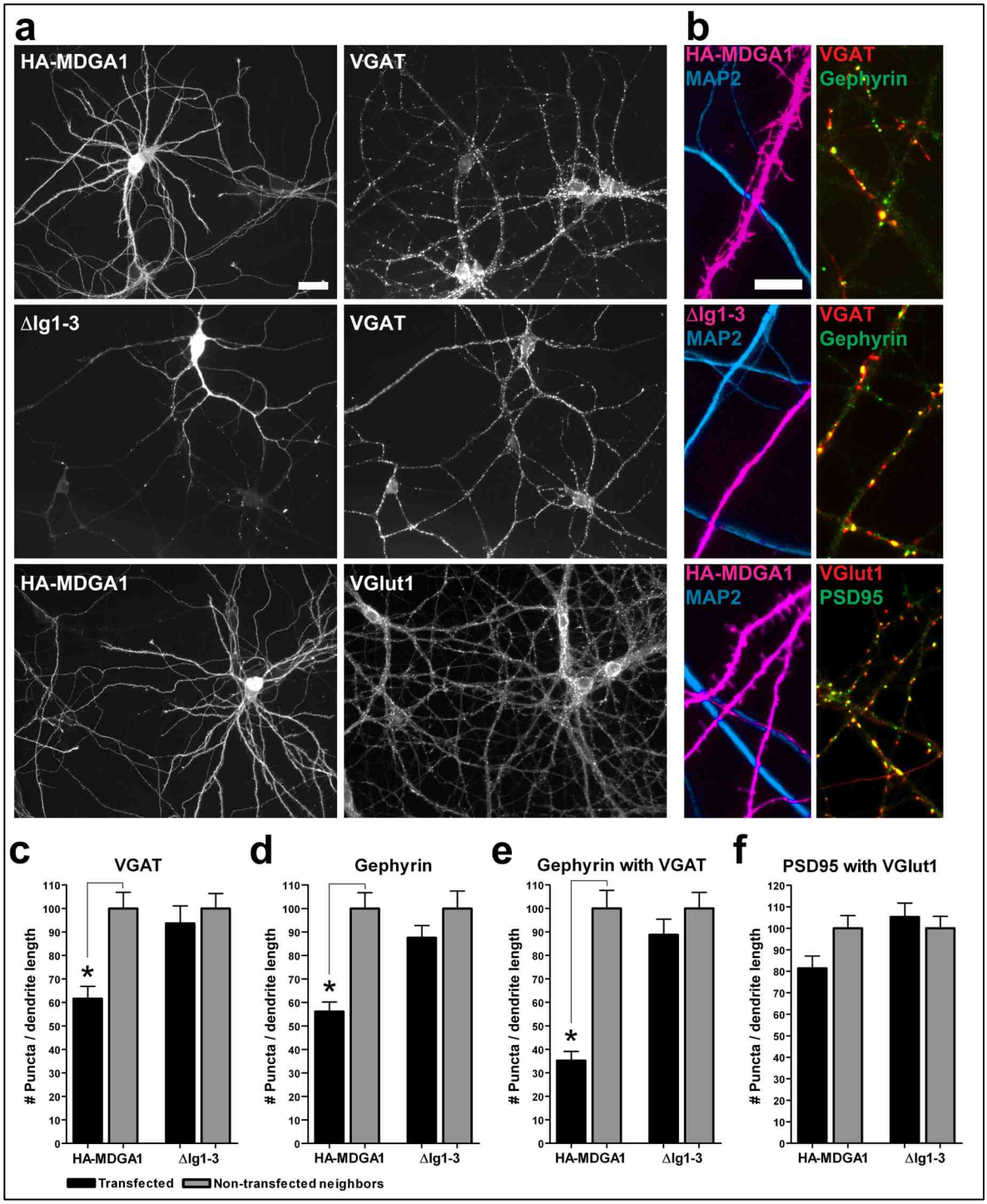
a
HA-MDGA1
VGAT
ΔIg1-3
VGAT
HA-MDGA1
VGlut1
b
HA-MDGA1
MAP2
VGAT
Gephyrin
ΔIg1-3
MAP2
VGAT
Gephyrin
HA-MDGA1
MAP2
VGlut1
PSD95
c
VGAT
d
Gephyrin
e
Gephyrin with VGAT
f
PSD95 with VGlut1
Puncta / dendrite length
HA-MDGA1
ΔIg1-3
Transfected
Non-transfected neighbors

Figure 3.5: MDGA1 Overexpression Reduces Inhibitory Synapse Density in Culture
Cultured hippocampal neurons were transfected at 8 – 9 DIV with HA-MDGA1, or ∆Ig1-3 as a negative control. Neurons were stained for synaptic markers at 14 DIV. Images at 25X show a decrease in the inhibitory presynaptic marker VGAT in neurons transfected with HA-MDGA1 (top row), but not with ∆Ig1-3 (middle row). Neurons transfected with HA-MDGA1 did not appear to have any changes in the excitatory presynaptic marker VGlut1 (bottom row). (b) 63X images show non-transfected dendrites (blue) alongside transfected dendrites (pink) (left column), beside the same field stained for synaptic markers VGAT or VGlut1 (red), and gephyrin or PSD95 (green). Dendrites transfected with HA-MDGA1 show a decrease in both VGAT and gephyrin staining compared to neighboring non-transfected neurons (top row). This decrease is not observed for neurons transfected with ∆Ig1-3 (middle row), or neurons transfected with HA-MDGA1 and stained for excitatory synaptic markers (bottom row). Scale bars are 30 µm in (a) and 10 µm in (b). (c - f) Quantitation of cluster density for VGAT (c), gephyrin (d), gephyrin with VGAT (marking inhibitory synapses) (e), and PSD95 with VGlut1 (marking excitatory synapses) (f) in neurons overexpressing HA-MDGA1 or negative control ∆Ig1-3. Data are normalized to non-transfected neighboring neurons (grey). ANOVA, $P < 0.0001$, n = 10 cells from three independent experiments; *$P < 0.001$ compared to non-transfected neighbors in post-hoc Bonferroni test for (c), (d), (e). ANOVA, $P = 0.0285$, n = 30 cells each for (f). Results are expressed as mean ± SEM.

3.3.6 Knockdown of MDGA1 Increases Inhibitory Synapse Number in Culture

We next tested the effects of reducing the levels of MDGA1 in cultured hippocampal neurons. We designed a short-hairpin RNA (shRNA) construct to knockdown MDGA1, which we confirmed in Western blots with co-transfected HEK cells and in cultured cortical neurons (Figure 3.6, e). Specificity for the sh-MDGA1 construct was also confirmed as MDGA2 levels in transfected HEK cells were not affected (Figure 3.6, e). Knockdown for endogeneous MDGA1 in cultured hippocampal neurons significantly increased the number of inhibitory synapses, compared to a control shRNA (sh-con) (Figure 3.6, a and b). These reductions were rescued by co-expression with an shRNA resistant form of MDGA1, MDGA1*. Knockdown of MDGA1 had no effect on the number of excitatory synapses (Figure 3.6, c and d). These data suggest that MDGA1 modulates the number of inhibitory synapses.

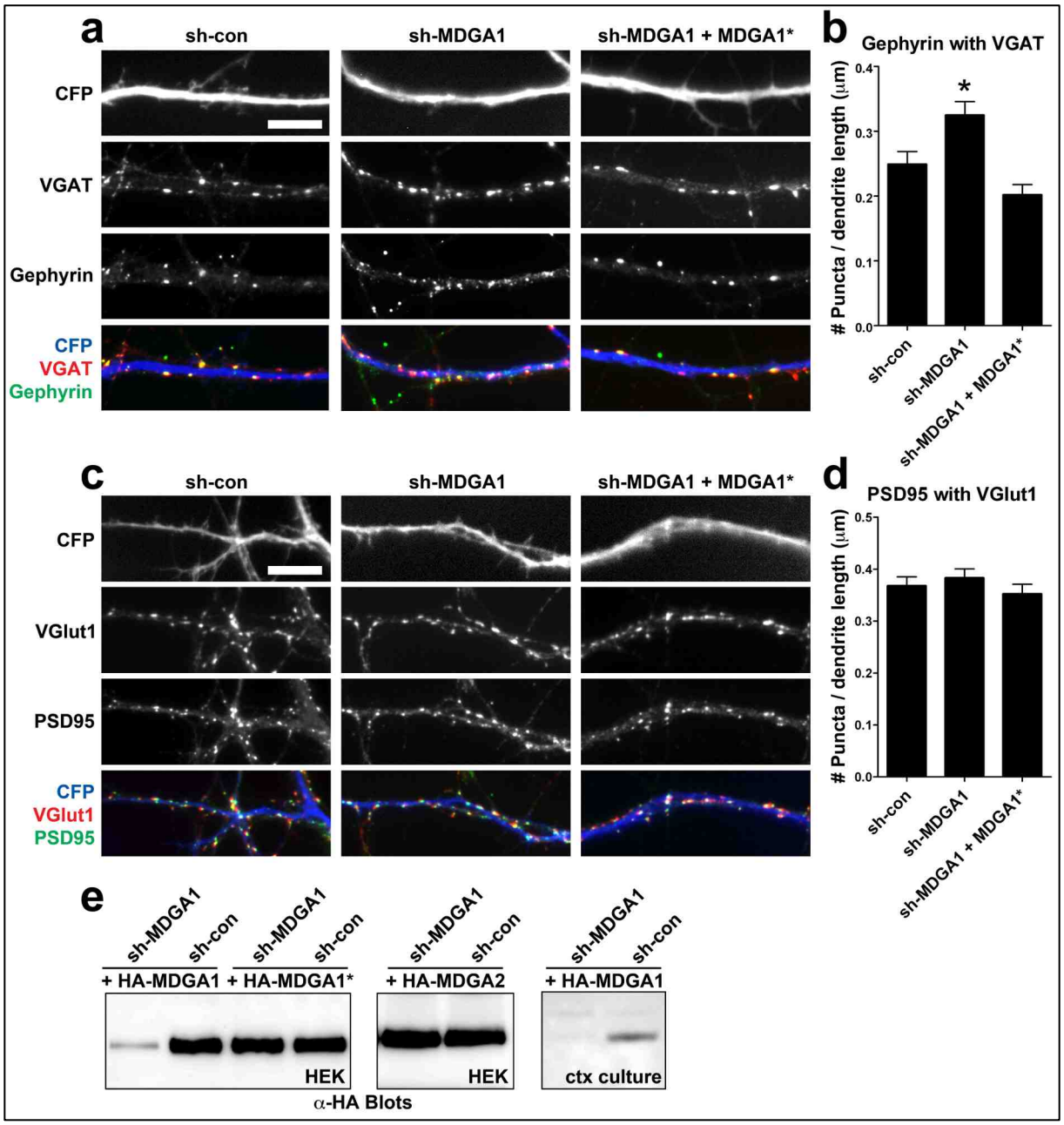
a
sh-con
sh-MDGA1
sh-MDGA1 + MDGA1*
CFP
VGAT
Gephyrin
CFP
VGAT
Gephyrin
b
Gephyrin with VGAT
Puncta / dendrite length (μm)
0.4
0.3
0.2
0.1
0.0
*
sh-con
sh-MDGA1
sh-MDGA1 + MDGA1*
c
sh-con
sh-MDGA1
sh-MDGA1 + MDGA1*
CFP
VGlut1
PSD95
CFP
VGlut1
PSD95
d
PSD95 with VGlut1
Puncta / dendrite length (μm)
0.5
0.4
0.3
0.2
0.1
0.0
sh-con
sh-MDGA1
sh-MDGA1 + MDGA1*
e
sh-MDGA1
sh-con
sh-MDGA1
sh-con
sh-MDGA1
sh-con
sh-MDGA1
sh-con
+ HA-MDGA1
+ HA-MDGA1*
+ HA-MDGA2
+ HA-MDGA1
HEK
HEK
ctx culture
α-HA Blots

Figure 3.6: MDGA1 Knockdown Increases Inhibitory Synapse Density in Culture
Cultured hippocampal neurons were transfected at 8 – 9 DIV with a vector expressing ECFP and short-hairpin RNA (shRNA) construct to knockdown MDGA1 (sh-MDGA1), a control shRNA (sh-con), or sh-MDGA1 with an sh-RNA-resistant form of MDGA1 (MDGA1*). Neurons were stained for synaptic markers at 14 DIV. (a) Small regions of dendrites showing CFP transfection (blue in bottom row), VGAT (red) and gephyrin (green). Inhibitory synapses are marked by gephyrin puncta apposed to VGAT puncta (yellow). (b) Quantitation of cluster density for gephyrin with VGAT. MDGA1 knockdown selectively increased the density of gephyrin with VGAT clusters (marking inhibitory synapses). This effect was rescued with co-expression of MDGA1*. ANOVA, $P < 0.0001$, n ≥ 10 cells from four independent experiments; $P < 0.05$ compared to sh-con in post-hoc Bonferroni test. (c) Small regions of dendrites showing CFP transfection (blue in bottom row), VGlut1 (red) and PSD95 (green). Excitatory synapses are marked by PSD95 puncta apposed to VGlut1 puncta (yellow). (d) Quantitation of cluster density for PSD95 with VGlut1. MDGA1 knockdown did not change the density of PSD95 with VGlut1 clusters (marking excitatory synapses). ANOVA, $P = 0.4784$, n = 10 cells each in four (excitatory measures) or five (inhibitory measures) independent experiments. Scale bars are 10 μm. Results are expressed as mean ± SEM. (e) Co-transfection of sh-MDGA1 with HA-MDGA1 in HEK cells showed a decrease in expression, but no decrease was seen with co-transfection with sh-con. HA-MDGA1* was not affected by sh-MDGA1 co-transfection (left gel). Neither sh-con or sh-MDGA1 decreased expression of HA-MDGA2 in co-transfected HEK cells (middle gel). Knockdown of MDGA1 was also confirmed in co-transfected cortical cultured neurons (ctx culture) (right gel).

3.4 Discussion

The formation and specification of synapses requires the coordinated efforts of a number of secreted and cell adhesion molecules to properly align the pre- and postsynaptic compartments and create functional synapses that can respond to activity. In this study, we have identified the MDGA family of proteins as new molecular players in synaptogenesis. We show that both MDGA1 and 2 bind neuroligin-2 with high affinity, with binding for MDGA1 occurring through the first three Ig-repeats (Figure 3.1). No binding was observed to neuroligin-1 in similar assays, suggesting that MDGAs may specifically act through neuroligin-2. However, preliminary results suggested that binding may occur between MDGAs and neuroligin-1 at high concentrations of Nlg1-Fc (not shown), but we were unable to demonstrate saturated binding. Therefore, it is possible that in the intact brain, there may be some situations where MDGAs are present in a high enough ratio to

neuroligin-1 to have a physiological effect on this synaptic protein as well. MDGA1 was found to partially co-localize to inhibitory synapses and with neuroligin-2 in culture, placing it in a relevant location to influence synapse development and maintenance (Figure 3.2). The fact that some MDGA1 was also localized at excitatory synapses suggests that it may have other synaptic functions, perhaps with the other neuroligins. It will be important in the future to assess potential interactions of MDGAs with neuroligin-3 and neuroligin-4. Neuroligin-3 was localized to both excitatory and inhibitory synapses and forms complexes in brain with neuroligin-1 and with neuroligin-2 (Budreck and Scheiffele, 2007). However, so far deficits in excitatory or inhibitory synaptic transmission have not been reported in mice lacking neuroligin-3 (Etherton et al., 2011; Tabuchi et al., 2007). Neuroligin-4 contributes to glycinergic inhibitory transmission in retina but is also broadly expressed in the brain (Hoon et al., 2011).

MDGAs also localized to axons and thus may have functions there as well. While the major function of the previously identified extracellular binding partner of neuroligins, neurexins, is in axons to promote synapse development, a secondary function in dendrites as a negative regulator has been suggested (Taniguchi et al., 2007). Both overexpression and knockdown studies (Figures 3.5 and 3.6) suggest that the major function of MDGA1 is to supress inhibitory synapse development in dendrites of cultured hippocampal neurons. However, it will be important to test MDGA1 and MDGA2 synaptic function in multiple circuits *in vivo*.

We demonstrated that MDGA1, through its Ig1-3 repeats, can specifically block the effects of neuroligin-2, but not neuroligin-1, in the presynaptic induction co-

culture assay (Figure 3.3). This inhibition by MDGA1 is likely accomplished through blocking the binding of neuroligin-2 to neurexins, and not through retention of neuroligin-2 in intracellular compartments (Figure 3.4). In further support of a specific interaction with neuroligin-2, which is known to modulate inhibitory synaptogenesis, we found a decrease in inhibitory, but not excitatory, synaptic markers when MDGA1 is overexpressed in hippocampal cultured neurons (Figure 3.5). Last, knockdown of MDGA1 in culture had the opposite effect by increasing inhibitory synapses, while having no effect on excitatory synapses (Figure 3.6). Together, these results suggest that MDGAs can modulate the number of inhibitory synapses through interacting with neuroligin-2. Further studies with MDGAs could focus on *in vivo* effects of MDGA overexpression or knockout; however, given the roles of MDGA1 in neuronal migration (Ishikawa et al., 2011; Takeuchi and O'Leary, 2006), care would have to be taken to examine synaptic effects in a knockout situation without incurring adverse migration deficits. With that in mind, it would be interesting to determine effects on synaptic composition and transmission, as well as behaviours, and compare these with phenotypes of neuroligin KO and knock-in (KI) mouse models.

The neuroligin family plays important roles in synapse development and maintenance. In humans, there are five neuroligins genes (Bolliger et al., 2001), while rodents have four neuroligins, which correspond with their human orthologs (Bolliger et al., 2001; Ichtchenko et al., 1996). Studies in rodents have shown that all neuroligins are found at postsynaptic densities, with neuroligin-1 and neuroligin-2 specifically localized to excitatory and inhibitory synapses, respectively, while neuroligin-3 is at both types of synapses (Budreck and Scheiffele, 2007; Song et al.,

1999; Varoqueaux et al., 2004). Furthermore, neuroligin-1 KO mice have specific deficits in excitatory neurotransmission, with no changes in inhibitory transmission, while neuroligin-2 knockout mice show a decrease in inhibitory neurotransmission with no change in excitatory (Chubykin et al., 2007). Since both single KO mice and a triple neuroligin-1, -2, -3 mouse all still form synapses, it appears that neuroligins play a role in synapse maturation and specification, rather than in initial synapse formation (Varoqueaux et al., 2006). In this capacity, neuroligins may help maintain the E/I balance in the brain. Recent studies with transgenic mice overexpressing neuroligins support this hypothesis. An increase in neuroligin-1 expression resulted in increased maturation of excitatory synapses, a shift in synaptic activity towards excitation, impairments in long-term potentiation (LTP) induction, and deficits in memory acquisition (Dahlhaus et al., 2010). In contrast, overexpression of neuroligin-2 altered the E/I balance towards inhibition and increased the frequency of miniature inhibitory synaptic currents. Interestingly, these neuroligin-2 overexpressing mice also exhibited impaired social interactions, anxiety, stereotyped behaviours and increased spiking activity (Hines et al., 2008).

These results are particularly interesting in light of the present study, as an increase in MDGA expression / activity could act to suppress neuroligin-2, thus increasing the effects of neuroligin-1 and shifting the E/I balance towards excitation. On the other hand, a decrease in MDGA expression / activity could effectively increase the actions of neuroligin-2, which would decrease the E/I ratio and could lead to similar behavioural abnormalities and seizure activity as seen in the neuroligin-2 overexpressing mice. The latter case seems particularly relevant when

considering that two independent studies have recently identified MDGA1 as a risk factor for schizophrenia (Kahler et al., 2008; Li et al., 2011), and another study linked MDGA2 to autism (Bucan et al., 2009), both of which are characterized by abnormal behaviours and possibly alterations in the E/I balance. In the case of MDGA1, SNPs were found in intronic regions, so it is difficult to determine what downstream effect these changes would have on expression level or function of MDGA1 protein (Kahler et al., 2008; Li et al., 2011), but given the results presented here, either an increase in MDGA1 or production of a defective protein product could have deleterious effects on synaptic function by upsetting the E/I balance. In the case of MDGA2, exonic deletions were detected which would result in truncation of the protein (Bucan et al., 2009). It will be important to determine the effect these disease-associated variants have on MDGA2 activity.

Interestingly, there is emerging evidence that suggests that both ASDs and schizophrenia may be caused, as least in part, by synaptic abnormalities (Abrahams and Geschwind, 2008; Betancur et al., 2009; Bourgeron, 2009; Ey et al., 2011; Faludi and Mirnics, 2011; Peca et al., 2011b; Sudhof, 2008). Mutations, CNVs and SNPs have been found in autistic patients in neurexin-1 (*NRXN1*) (Bucan et al., 2009; Feng et al., 2006; Szatmari et al., 2007), neurexin-2 (*NRXN2*) (Gauthier et al., 2011), neuroligin-1 (*NLGN1*) (Glessner et al., 2009), neuroligin-3 (*NLGN3*) and neuroligin-4 (*NLGN4*) genes (Jamain et al., 2003; Laumonnier et al., 2004; Lawson-Yuen et al., 2008). Furthermore, one of the LRRTM family of newly reported binding partners for neurexins (*LRRTM3*) (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010), the SHANK family of synaptic scaffold proteins which bind indirectly with

neuroligins (*SHANK2, SHANK3*) (Sheng and Hoogenraad, 2007), the synaptic CAMs contactins 3 and 4 (*CNTN3, CNTN4*) (Karagogeos, 2003), the contactin-associated proteins which are similar in structure to neurexins (*CNTNAP2*) (Pillai et al., 2007), the MAGUK protein CASK which binds neurexins (Hata et al., 1996), and other synapse-associated proteins have all been linked to ASDs and/or intellectual disability as well (Berkel et al., 2010; Betancur et al., 2009; Durand et al., 2007; Glessner et al., 2009; Li et al., 2011; Moessner et al., 2007; Morrow et al., 2008; Pinto et al., 2010; Roohi et al., 2009; Sousa et al., 2010; Sudhof, 2008). In addition, *NRXN1, NRXN2*, *LRRTM1*, *SHANK3*, *CNTN5*, *CNTNAP1* and *CNTNAP2* have been linked to schizophrenia (Francks et al., 2007; Gauthier et al., 2010; Gauthier et al., 2011; Kirov et al., 2008; Pickard, 2011), suggesting there may be similar underlying causes or risk factors for schizophrenia and ASDs. The discovery of contactins as autism risk factors is especially interesting since, like MDGAs, contactins are GPI-linked members of the Ig superfamily of CAMs (Karagogeos, 2003), and these two families of proteins share ~24% homology (Bucan et al., 2009). Thus, MDGAs, which we show here to be present at synapses and bind neuroligin-2 to elicit specific effects at inhibitory synapses, join a wide range of other synaptic proteins linked to neurological diseases.

These mutations in synaptic proteins are often very rare mutations in the population, each of which confer a high increase in risk for the disease, as opposed to previous theories which assumed that common variants would account for most disease incidence by increasing risk by a small amount (State and Levitt, 2011). The fact that the discovery of these rare variants for numerous neurodevelopmental

diseases appears to be converging on synaptic pathways offers intriguing possibilities for further study. Some progress has been made in modeling autism-linked mutations in mice. Neuroligin-3 R451C knock-in mice, which mimic a human neuroligin-3 mutation found in ASDs, have moderate social deficits and an increased spatial learning capacity, as well as increased density of GABA synapses, and increased inhibitory synaptic transmission in the somatosensory cortex (Tabuchi et al., 2007). Importantly, these changes were not observed in neuroligin-3 KO mice, suggesting a unique gain-of-function effect, and suggest that changes in the E/I balance may be involved in ASD-like behaviours (Tabuchi et al., 2007). In addition, neuroligin-4 KO mice show impairments in social interactions and communication (Jamain et al., 2008). Further studies with knock-in mice duplicating disease variants found in neurodevelopmental disorders will be useful to determine the precise contribution that each of these molecular players has in synapse development and maintenance.

In conclusion, we have identified a new synaptic function of the MDGA family of proteins in which they can suppress the actions of neuroligin-2 and influence inhibitory synapse formation or maintenance. These actions on neuroligin-2 may shift the E/I balance in the brain, which may play a role in neurodevelopmental disorders such as autism and schizophrenia. It will be important to further characterize MDGAs and other synaptic CAMs that are genetically linked to neurodevelopmental disorders to better understand how synaptic changes can lead to complex behavioural phenotypes, and to eventually develop customized treatment options.

Chapter 4: Discussion and Conclusions

The brain contains billions of neurons, all of which must establish specific connections with a number of synaptic partners. These patterns of connections form the functional circuits that allow for the production of complex behaviours, which can be modified in response to activity and learning. Thus, the mechanisms by which synapses develop and mature are critical biological processes. Synaptogenesis is a multi-step process involving a number of molecular players. Cell adhesion molecules play a particularly important role in inducing bi-directional, trans-synaptic signaling to mediate the differentiation and maturation of pre- and postsynaptic compartments. Although a number of such cell adhesion molecules have been characterized, phenotypes from knockout mouse studies suggest that there are still undiscovered synaptic organizing molecules. Thus, the overarching goal of this work was to discover and characterize new molecules that can influence synapse development. The results of this study provide evidence for a new synapse-promoting molecule (calsyntenin-3), and a new negative regulator of synapses (MDGAs). These results were largely obtained using cultured primary hippocampal neurons as a model system for studying synaptogenesis.

4.1 The Hippocampal Neuron Culture System

Both studies presented here (Chapters 2 and 3) rely heavily on the manipulation and analysis of cultured hippocampal neurons. Preparations of hippocampal cultures have been well-characterized and afford a number of advantages, but also have some limitations. The clear advantage of culture

preparations over brain slice preparations or whole brain is the fact that cultures can be readily manipulated and observed, and are also far less complex. The cultures used here are “sandwich-style” cultures, in which primary dissociated hippocampal neurons are dissected from late stage rat embryos, cultured at a low density on polylysine-coated glass coverslips and suspended over a glial feeder layer for trophic support (Kaech and Banker, 2006). Compared to “mixed” cultures in which glia and neurons are plated together, sandwich-style cultures allow for clearer visualization of neuron morphology and synaptic staining as glia are not in the same plane of view; neurons are growing on coverslips which can be removed from the dish for staining, while glia are growing on the dish itself. Cultured neurons are a great model system because development of neuronal polarity and synaptogenesis appear to proceed in cultures with a similar time course and by similar mechanisms as they do *in vivo* (Kaech and Banker, 2006). For example, cultured hippocampal neurons develop axons and dendrites that form regular axo-dendritic synapses; these can be identified by immunostaining for synaptic vesicle markers and display endocytic recycling just like synapses *in vivo* (Bartlett and Banker, 1984; Matteoli et al., 1992). Numerous contacts form between synaptic specializations as early as 3 days in culture and include presynaptic vesicle clusters, while complete synapses require postsynaptic maturation and begin to appear at ~5 days in vitro (DIV) (Banker and Goslin, 1998; Fletcher et al., 1994). Dendrite growth and synaptogenesis is highest during the second and third weeks in culture (Kaech and Banker, 2006). The time of dissection (usually embryonic day 18) ensures that the culture will consist of mainly pyramidal neurons that form excitatory synapses with

each other (Banker and Cowan, 1977), while approximately 7% of neurons are GABAergic interneurons that form synapses with each other and also preferentially on pyramidal cell bodies, similar to the situation *in vivo* (Benson et al., 1994). The homogeneity of cell types in hippocampal cultures is an advantage over cultures from other brain regions, which can contain many subtypes of neurons.

Despite the versatility of the culture system, there are clearly a number of limitations as well. Dissociated cultures lack the cytoarchitecture found in intact tissue, thus some aspects of development, such as the formation of unidirectional synaptic circuits in the hippocampus, may not be completely recapitulated. However, recent work suggests that, even in culture, there are several aspects of specificity that are still preserved, such as preferential formation of dentate gyrus – CA3 pyramidal cell synapses (Williams et al., 2011). In addition, although the segregation of glia and neurons offers excellent imaging opportunities, it is also somewhat artificial as glia and neurons are mixed *in vivo*, so there may also be aspects of development requiring physical association of glia and neurons that are not recapitulated in sandwich-style cultures. Another drawback of using primary neuron cultures is that they are inherently variable and very sensitive. Despite strict adherence to a standard protocol, sometimes hippocampal cultures will simply not develop as robustly as usual; thus it is critical to only use “good” cultures for analysis. Last, it is clear that, at least in the field of synaptogenesis, candidate proteins may have very different functions in culture and *in vivo*, as in the latter case there is the opportunity for much more complex regulation. It is therefore imperative that findings from cell culture experiments be validated and further investigated in

whole animal models when possible. Nevertheless, primary neuron culture experiments have allowed for the identification and detailed characterization of a number of important aspects of synaptic development, and continue to be an important model for studying this complex developmental process.

4.2 Calsyntenins

4.2.1 Overall Conclusions

Calsyntenin-3 was isolated from an un-biased expression screen to identify proteins able to induce presynaptic specializations in contacting axons. It is interesting to note that this type-1 transmembrane protein does not share synaptogenic activity with the other two calsyntenin family members, as assessed in hippocampal and cortical co-cultures. Characterization of the induced presynaptic specializations showed that they have recycling vesicles and are both glutamatergic and GABAergic in nature. In addition, both the extracellular cadherin and LNS domains are important for presynaptic induction. Overexpression of the ΔPCS version of calsyntenin-3 increased presynaptic protein clustering, while dispersing postsynaptic clusters. Initial binding assay results suggest that calsyntenin-3 binds neurexin-1α with high affinity. One of the strengths of these findings is the fact that calsyntenin-3 was found in an un-biased functional screen which identifies the most potent inducers of synaptogenesis. In comparison, neuroligin-3 was isolated in a negative pool in the same screen (Linhoff et al., 2009), although it can also induce synaptogenesis in culture (Chih et al., 2004). The additional co-culture assays provide clear evidence for a synaptic function of calsyntenin-3, although further work

will be needed to determine exactly how calsyntenin-3 instructs synaptic development *in vivo* (see Section 4.2.2.3 below).

Despite sharing a highly similar extracellular domain with calsyntenin-3, calsyntenin-1 and -2 did not have synaptogenic activity in the assays used here. The low level of surface expression of full length calsyntenin-1 and calsyntenin-2 could have been responsible for the apparent lack of activity; however, surface-expressed extracellular domains of calsyntenin-1 and -2 tethered to the membrane by the CD8 transmembrane domain still did not induce presynaptic differentiation. In comparison, the same truncation for calsyntenin-3 had an equivalent level of activity as full-length calsyntenin-3, suggesting that the intracellular domain is not required for presynaptic induction. In co-culture assays, this finding makes sense as an intracellular domain-mediated mechanism would have to act through proteins endogenously expressed in COS cells, and COS cells are unlikely to express a synapse-specific postsynaptic protein able to co-mediate synaptic induction. Nevertheless, it is possible that, in the brain, the intracellular domains of calsyntenin-1 and/or -2 are important for some sort of synaptic function. Like calsyntenin-3, analysis of function and expression patterns *in vivo* may be the best way to study this possibility.

There are a few limitations to the results described in Chapter 2. Previous work reports that calsyntenins are postsynaptically localized (Hintsch et al., 2002; Vogt et al., 2001), but this finding could not be replicated in the hippocampal culture system used here due to a lack of good antibody for immunostaining, as well as the fact that the vast majority of calsyntenin-3 appears to be cleaved in culture.

However, initial results from mouse studies in our lab show that only about 50% of calsyntenin-3 is cleaved in whole brain, suggesting that cleavage mechanisms are upregulated in culture for some reason. This high level of cleavage also made overexpression studies difficult, which is why the deletion construct ΔPCS was utilized instead of full length calsyntenin-3. Generally, overexpression studies are complimented by a knockdown approach; however, since there appears to be so little full-length calsyntenin-3 expressed in cultured neurons (only ~ 10%), this approach was not feasible.

In addition, the evidence for synaptogenic function of calsyntenin-3 shown here is derived largely from the results of immunostaining experiments; functional correlates to synaptic activity have not yet been determined. Although calsyntenin-3-induced presynaptic specializations do show synaptotagmin uptake, suggesting the formation of functional neurotransmitter release sites, further studies are needed to show what functional changes in synapses may accompany calsyntenin-3 expression. Functional correlates could most easily be obtained by using electrophysiological methods in calsyntenin-3 overexpressing neurons in culture. Results from such experiments may resemble those for neuroligins, in which overexpression in culture can induce increases in both mEPSC and mIPSC frequency and/or amplitude (Dean and Dresbach, 2006), or may reveal different effects on synapse function or plasticity. Alternatively, electrophysiology could be used *in vivo* (see 4.2.2.3 below) to determine possible roles for calsyntenin-3 in synaptic transmission and plasticity, including the potential for differential functions at excitatory versus inhibitory synapses.

There are a number of other aspects of synaptogenic activity of calsyntenin-3 that could be further investigated. The fact that overexpression of calsyntenin-3 dispersed postsynaptic protein clusters suggests that it may be capable of bi-directional signaling to instruct postsynaptic differentiation. Calsyntenin-3 (both wild type and ΔPCS) with an extracellular YFP tag has recently been cloned in our lab; this could be transfected into neurons and directly aggregated on dendrite surfaces using anti-YFP antibodies attached to beads. Accumulation of postsynaptic proteins at sites of calsyntenin-3 aggregation would suggest that it can signal bi-directionally. Comparing this result to a YFP-tagged version of calsyntenin-3 lacking the intracellular domain (such as Myc-C3EXTM-CFP) could prove that postsynaptic protein clustering occurs via the intracellular domain of calsyntenin-3, rather than possible binding to an extracellular co-factor. Such an approach has been used previously to demonstrate direct postsynaptic induction by neuroligins (Graf et al., 2004). If the intracellular domain of calsyntenin-3 does not appear to be necessary for clustering of postsynaptic proteins, this would suggest binding of an extracellular co-factor which itself signals intracellularly to mediate postsynaptic differentiation. Various approaches could be used to search for such a partner, such as yeast-two-hybrid screens using the extracellular domain of calsyntenin-3 as bait, anti-calsyntenin-3 immunoprecipitations from brain with mass spectroscopy analysis of bound proteins, or screening of candidate proteins in COS cells by assaying binding of Clstn3-Fc protein.

However, if it appears that calsyntenin-3 can directly induce postsynaptic protein clustering via the intracellular domain, further analysis involving similar co-

immunoprecipitations and other assays of binding may help to identify potential direct or indirect intracellular postsynaptic binding partners. Binding to Mint2/X11L has already been reported (Araki et al., 2003), so it is possible that binding to Mint adaptor proteins is involved in protein aggregation, or other intracellular binding partners may exist. Calsyntenins also apparently bind calcium via the intracellular domain (Hintsch et al., 2002; Vogt et al., 2001), so it is possible that calcium modulates intracellular signaling or binding; this is another potential area for study.

In addition, given the fact that full-length or membrane-anchored, but not secreted, calsyntenin-3 induces presynaptic specializations, it is tempting to hypothesize that the secreted portion may act as an inhibitory signal. Although co-culture tests in which COS cells were co-transfected with various ratios of secreted and full-length calsyntenin-3 did not reveal an inhibitory affect for the soluble extracellular domain, it is possible that further varying of experimental conditions (such as further increasing the amount of soluble calsyntenin-3) or other approaches may yield positive results. Given the binding to presynaptic neurexins, a mechanism by which cleaved, soluble calsyntenin-3 binds to neurexin to block binding of membrane-anchored calsyntenin-3 could be an interesting way to regulate synaptic development or maturation. Cleaved calsyntenin-3 could also potentially block binding of neurexin to other postsynaptic partners like LRRTMs and neuroligins, and thus have more wide-ranging effects at synapses; this is a particularly intriguing idea for study using the co-culture and neuron culture systems.

In my opinion, there are three major areas in which the synaptogenic ability of calsyntenins could be further investigated: regulation of proteolytic cleavage, binding

to neurexins, and analysis of functions *in vivo*. These possibilities will be described below in Section 4.2.2.

4.2.2 Future Directions

4.2.2.1 Regulation of Calsyntenin Cleavage

When calsyntenin-3 was first pulled out of the un-biased expression screen, the idea that cleavage could potentially regulate the synaptogenic activity was a very unique and interesting avenue to consider. However, this aspect of calsyntenin-3 function has been particularly difficult to address. Once it was clear that calsyntenin-3 had to be membrane-anchored to exert synaptogenic effects, the idea of blocking cleavage to increase full length calsyntenin-3, and hopefully synaptogenic activity, became the obvious avenue to pursue. At the time, ADAM10 and ADAM17 cleavage of calsyntenins had not yet been reported, so a variety of different classes of protease inhibitors were tested in cortical cultures and the amount of cleaved and full length endogenous calsyntenin-3 was assayed. The intent was to find an inhibitor that would block or greatly reduce proteolytic processing of calsyntenin-3, then use this inhibitor in hippocampal cultures to assay possible effects at synapses. Unfortunately, despite extensive modification of experimental conditions, no effective inhibitor was ever found.

However, the proteolytic processing of calsyntenin-3 still remains an interesting question. Calsyntenins can be cleaved by both ADAM10 and ADAM17 (Hata et al., 2009), which are matrix metalloproteases expressed mostly in neurons and glia, respectively (Yang et al., 2006). ADAMs have been studied in detail and, due to their ability to mediate both cell adhesion and the proteolytic release of a

diverse set of substrates, they are implicated in a wide range of cellular processes in both health and disease (Reiss and Saftig, 2009). In the central nervous system specifically, ADAMs seem to be involved in almost every developmental process, including cell proliferation, migration, differentiation, neurite remodeling, synaptic plasticity and learning and memory (Lee et al., 2008b; Reiss and Saftig, 2009; Yang et al., 2006). These multiple roles are not surprising considering that proteolytic cleavage of transmembrane proteins can disrupt cell-cell interactions, but can also release bio-active secreted domains and C-terminal domains involved in intracellular signaling and regulation of gene transcription (Lee et al., 2008b). Furthermore, there is a plethora of ADAM10 and ADAM17 substrates in the CNS, including N-cadherin, APP, Notch, ephrinA2 and A5, L1, NCAM, NPR, TrkA, and PTP-LAR (Reiss and Saftig, 2009). The situation is further complicated by the fact that ADAMs themselves are regulated by a number of developmental and environmental cues, including growth factors, intracellular calcium concentrations and neuronal activity; furthermore, this regulation can take place at a number of different levels, including transcription, translation, alternative splicing, changes in protease stability, cellular localization and interaction with other proteins (Huovila et al., 2005; Reiss and Saftig, 2009).

Despite these complexities, regulation of other synaptic CAMs by ADAM processing has been reported (Juttner et al., 2005; Maretzky et al., 2005; Reiss et al., 2005; Uemura et al., 2006), and perhaps similar methods could be used to address the physiological significance of calsyntenin-3 cleavage. For example, cultures from ADAM10 or 17 KO mice could be used to examine changes in the

amount of full length calsyntenin, and possible effects on synapse number. Since shedding can also be regulated by activity, comparing synaptic effects of activity manipulations (alongside levels of calsyntenin cleavage) in ADAM KO mouse cultures versus wild type cultures may be instructive. However, given the number of other synapse-associated substrates for ADAMs, results from these types of assays would still be indirect evidence and may be complicated by off-target effects relating to changes in processing of other substrates. The best available proof may be to simply correlate changes in ADAM-mediated calsyntenin-3 processing to changes in synapses, while acknowledging that ADAMs do have other substrates. If this hypothesis could be supported, along with the results presented in Chapter 2 showing that membrane-anchored, but not secreted, calsyntenin-3 mediates presynaptic induction, this would be fairly strong evidence for a role of regulated cleavage in calsyntenin-3 activity.

Another aspect to consider is the reported coordinated metabolism of calsyntenins and APP. APP is a well-characterized substrate for extracellular proteases, and recent evidence shows it can also induce presynaptic specializations (Wang et al., 2009). Thus, another interesting avenue to pursue is the possibility that APP and calsyntenin-3 may act in concert to mediate synaptogenesis, or may have additive effects. This hypothesis could be initially tested by co-transfection of calsyntenin-3 and APP in co-culture assays. If they indeed appear to cooperate, determining how cleavage of one affects activity of the other could be another long-term avenue of experimentation to pursue. The interaction of calsyntenin-3 and APP may also have physiological significance later in development. A recent study

reported that calsyntenin-3 is upregulated by treatment with Aβ peptides, and overexpression of calsyntenin-3 in cortical neurons increases their susceptibility to cell death (Uchida et al., 2011). It is unclear why this could be the case, but perhaps calsyntenin-3 levels are tightly regulated by APP, and a decrease in APP results in an over-compensation by calsyntenin-3. However, proteolytic cleavage may also play a role, as an increase in calsyntenin-3 may translate to an increase in the secreted domain. If this domain can have inhibitory effects on synapse formation or stability, as hypothesized above, then perhaps an increase in calsyntenin-3 in this system results in an overall decrease in synaptic density, which may trigger other downstream pathways that increase the likelihood of cell death. A better understanding of the interplay between APP, calsyntenin-3 and regulation of proteolytic processing is clearly needed to determine the synaptic outcomes that may result.

4.2.2.2 Calsyntenin-3 as a Neurexin Ligand

The surprising finding that calsyntenin-3 binds with high affinity to neurexin-1α opens up many areas for immediate experimentation; some of these areas are currently being pursued by other members of the Craig laboratory. The first obvious question is whether calsyntenin-3 equally binds all neurexins, or if there is an isoform- or splice-specific code. Initial results from our laboratory using binding assays with Clstn3-Fc protein suggest that calsyntenin-3 binds all three α-neurexins, but does not bind β-neurexins. Furthermore, this binding appears to be unaffected by the presence of neurexin splice site 4 (SS4) (Craig Lab Research Assistant Lin Luo, unpublished results). These binding assays appear to be confirmed in co-culture

recruitment assays, in which Myc-C3ΔPSC-CFP in neurons was recruited to sites of contact with COS cells expressing α-neurexins(±SS4) but not β-neurexins(±SS4) (Craig Lab PostDoc Dr. Tabrez Siddiqui, unpublished results). These initial tests could also be confirmed by showing a physical interaction using co-immunoprecipitation experiments. These preliminary results suggest that calsyntenin-3 binds neurexins with a different code than other neurexin binding partners, such as neuroligins, LRRTMs and Clbn-GluRδ2. Thus, evidence is mounting that neurexins may act as master organizers of synapses in the CNS.

Presumably, calsyntenin-3 induces presynaptic differentiation via binding to presynaptic neurexins. Thus, it is likely that the domains needed for synaptogenic activity are the same needed for binding to neurexins. Binding assays with an α-neurexin-Fc fusion protein and COS cells transfected with various calsyntenin-3 domain mutants will help to answer this question, although given the results from co-cultures, it may be that both cadherin and LNS domains are needed for binding. Likewise, it will also be interesting to determine which LNS and/or EGF domains of α-neurexins are required for calsyntenin-3 binding; since calsyntenin-3 does not bind β-neurexins, it is unlikely that the LNS6 domain is involved. The fact that two point mutations in the calsyntenin-3 LNS domain resulted in large reductions in co-culture activity suggests that this domain is very important. Interestingly, one of these mutations, the DN/AA mutation, was chosen based on predicted similarity to calcium-coordinating residues in other LNS domains; the reduction observed with this point mutation suggests that calcium-binding may also be involved in binding to neurexins. Initially, this hypothesis could be tested quite simply by doing a binding

assay in calcium-free binding buffer. If binding is calcium-dependent, more sensitive techniques such as crystallization could be used to determine the sites of calcium coordination in the neurexin-calsyntenin complex. These results could also be corroborated by comparing binding of α-neurexin-Fc to COS expressing the DN/AA mutant compared to wild type calsyntenin-3. The second LNS point mutation, Q/K, was chosen based on similarity of a point mutation in *C. elegans* CASY-1 that is associated with learning deficits (Ikeda et al., 2008). If this mutant also exhibits reduced binding to neurexin, it would be interesting to find out if CASY-1 may bind *C. elegans* neurexin orthologs as well, or, on the other hand, if the learning and memory function of CASY-1 is unrelated to calsyntenin-3 / binding to neurexins. Cloning of additional calsyntenin-3 point mutations could be used to specifically pinpoint residues needed for calsyntenin-3 binding to neurexin / synaptogenic activity, similar to what has been done for neurexins and neuroligins (Graf et al., 2006). Using an α-neurexin-Fc fusion protein, neurexin binding to calsyntenin-1 and calsyntenin-2 could also be tested. Given that these two family members do not induce presynaptic differentiation in culture, it is unlikely that they bind neurexins. Perhaps analysis with knockout mice (see Section 4.2.2.3 below) will help shed light on what functions calsyntenin-1 and -2 have in the brain.

Once these structural questions are answered, more functional issues can be addressed: namely, if the synaptogenic activity of calsyntenin-3 is directly mediated by neurexins. For example, purified calsyntenin-3 could be attached to beads and presented to axons to determine if this is sufficient to cluster neurexins and other presynaptic proteins. Another idea would be to see if soluble α-neurexin can block

presynaptic induction by calsyntenin-3. Using mouse KO tissues could also prove useful; for example, perhaps the overexpression effects of calsyntenin-3 would be abolished in α-neurexin KO mouse tissues. These types of experiments would provide evidence for trans-synaptic signaling between calsyntenin-3 and α-neurexin during synapse development and/or maturation.

4.2.2.3 Analysis of Calsyntenin Function *In Vivo*

Ultimately, the true test for of synaptogenic proteins is the generation of knockout (KO) mouse models. Interestingly, some of the strongest "inducers" of synapses in culture appear to be more involved in synapse maturation rather than formation *in vivo*. For example, both triple neuroligin1-3 KO mice and α-neurexin-1-3 KO mice have severe deficits in neurotransmission but still form synapses (Missler et al., 2003; Varoqueaux et al., 2006). For calsyntenin-3, the generation and characterization of a knockout mouse would provide the best proof for a synaptic function *in vivo*. Although calsyntenin-1 and -2 appear to be negative in our co-culture assays, generation of KO mice for these family members may also provide evidence for a synaptic function in a specific brain region and/or developmental time window, or alternatively may suggest that they have very different roles than calsyntenin-3 *in vivo*. The generation of conditional knockouts would be particularly useful for studies involving the crossing of single calsyntenin knockouts to create multiple knockouts, for creating region-specific knockouts, and for crossing with other knockout mouse lines. Before analysis of knockouts, it would be important to examine more closely the developmental expression patterns of each calsyntenin family member in the brain (including in specific brain regions) in order to target

analysis to developmental stages/brain regions with high expression. Similar to the characterization of other KO mice, a multi-dimensional approach would be best. Briefly, this could include confocal microscopy and immunohistochemistry to examine synaptic density, subcellular fractionation to examine the levels and distribution of synaptic proteins, electrophysiology to examine changes in synaptic transmission, and various behavioural assays.

It is difficult to predict what the results of these assays may be, but it is likely that, like other “inductive” synaptic CAMs, knockout of just calsyntenin-3 may not result in drastic changes in the number of synapses. Rather, the phenotype may be very subtle. Considering that calsyntenin-3 can influence both excitatory and inhibitory synaptogenesis in culture, it is possible that changes in synaptic number and/or strength may be observed for both glutamatergic and GABAergic synapses. Given the link of calsyntenin-2 and the C. elegans ortholog CASY-1 to learning and memory (Hoerndli et al., 2009; Ikeda et al., 2008; Jacobsen et al., 2009; Papassotiropoulos et al., 2006; Preuschhof et al., 2010), it would be interesting to determine the behavioural characteristics of calsyntenin KO mice in tests of learning and memory. The full characterization of calsyntenin-1 and calsyntenin-2 mice would hopefully reveal the possible physiological roles of these family members – perhaps they do influence synapse function but in a specific subset of neurons or during a specific developmental window that could not be represented in the hippocampal culture system. Alternatively, these two proteins could have non-synaptic roles.

Given the reported role of calsyntenin-1 as a cargo docking protein (Araki et al., 2007; Konecna et al., 2006; Ludwig et al., 2009), it would be important to note if calsyntenin-1 KO mice display any signs of disrupted protein trafficking.

In the long term, calsyntenin KO mice (particularly the “synaptogenic” calsyntenin-3) could be crossed with other KO mice lines. As more neurexin binding partners are discovered, it seems that there may be some redundancy in CAMs that are important for synaptic development. Thus, a combined and targeted KO of calsyntenin-3, neuroligin(s) and LRRTM(s) may yield interesting new phenotypes and shed light on how these molecules may act in redundant vs. independent ways to facilitate synaptogenesis. Since calsyntenins have also been linked to APP metabolism and Alzheimer’s disease, another interesting avenue of study could be to cross calsyntenin KO mice with mouse models of AD, especially those expressing mutant APP variants. Analysis of such mice during aging and onset of AD-like symptoms may yield new insight on how calsyntenins and APP interact both in health and during disease.

4.3 MDGAs

4.3.1 Overall Conclusions

In Chapter 3, evidence is provided to show that MDGAs are synaptic modulators of neuroligin-2. Both MDGA1 and MDGA2 specifically bind neuroligin-2, but not neuroligin-1, with high affinity. This binding is mediated through the first three immunoglobulin (Ig) repeats in MDGA1. MDGA1 partially localizes at synapses with neuroligin-2. MDGA1 also inhibits the ability of neuroligin-2 to induce presynaptic

specializations in cultured neurons, most likely by blocking binding to neurexins, rather than affecting neuroligin surface expression. Overexpression of MDGA1 decreases inhibitory synapse formation in cultured neurons, while knockdown has the opposite effect and increases inhibitory synapses. These results are the first example of a negative-regulator for neuroligins, which have well-established roles in synaptic development and maturation. They also support the idea that, in addition to "synaptogenic" molecules, there are likely also "anti-synaptogenic" molecules which help regulate when and where synapses form. Another example of an anti-synaptogenic factor is the Wnt family, which control precise synapse placement in C. elegans motor neurons (Klassen and Shen, 2007). However, Wnts appear to act through intracellular signaling pathways, while MDGAs appear to directly block neuroligin binding to neurexin by binding neuroligins themselves. Furthermore, as MDGAs are GPI-linked proteins, they would need an additional co-factor to signal to the intracellular space.

The work described in Chapter 3 has a number of strengths. The direct binding between MDGAs and neuroligin-2 demonstrated in cell-based assays was shown to have synaptic effects both in co-culture assays and in cultured neurons. The specific decrease in inhibitory synapses with overexpression is complemented with the increase in inhibitory synapses with knockdown, which was rescued by a resistant form of MDGA1. These results are also consistent with the fact that neuroligin-2 plays a more dominant role at inhibitory synapses (Chubykin et al., 2007; Hines et al., 2008; Varoqueaux et al., 2004). One of the weaknesses of this study was the lack of antibody against MDGA1 or 2. With a good antibody, the

endogenous subcellular localization of MDGAs could be examined, and efficacy of knockdown could be directly measured. Antibodies can also be useful for western blotting and co-immunoprecipitation experiments, and could also be used for more precise localization using electron microscopy. Another weakness, similar to the work on calsyntenins, is the lack of functional data to correlate the changes in synaptic protein immunostaining with possible changes in neuronal activity. A simple way to address this issue would be to measure synaptic transmission properties in MDGA1 overexpressing and MDGA1 knockdown cultured neurons. Given the specific effects observed at inhibitory synapses in the immunostaining experiments shown here, electrophysiology might be expected to also reveal specific changes in inhibitory, but not excitatory, neurotransmission, such as decreases in mIPSC frequency and/or amplitude with MDGA1 overexpression and increases with MDGA1 knockdown.

In my opinion, there are three main areas that could be investigated to further expand upon the results presented in Chapter 3: further characterization of neuroligin-MDGA binding, analysis of synaptic functions of MDGA *in vivo*, and investigation of the association between MDGAs and neurodevelopmental disorders. These areas will be described in detail below.

4.3.2 Future Directions

4.3.2.1 Characterization of Neuroligin-MDGA Binding

The results presented in Chapter 3 show that binding of MDGA1 to neuroligin-2 is mediated by the first three Ig repeats in MDGA1. However, it is not known which residues of neuroligin-2 are necessary for MDGA1/2 binding. If an MDGA1-Fc fusion

protein were developed, similar binding assays could be done with neuroligin-2 deletion constructs to determine which domain(s) are needed. Co-immunoprecipitations could also be used to investigate binding, providing tagged constructs were used and/or good antibodies exist. Once this was determined, smaller point mutations could be cloned for both MDGAs and neuroligin-2 to pinpoint the essential residues needed for binding. These findings could be compared to the large body of data describing the binding determinants for neurexin binding to neuroligins, which would hopefully shed light on the molecular mechanism by which MDGA can block neuroligin binding to neurexin. Results from these types of studies may also suggest why MDGAs bind specifically to neuroligin-2 but not neuroligin-1; in this work, binding to neuroligin-3 and -4 was not determined, but this could also be easily tested with the development of MDGA-Fc fusion proteins.

MDGAs may block binding to neurexins by directly binding to the neurexin-binding face of neuroligin-2. Alternatively, MDGAs may bind at a different location / face than neurexins but binding could induce a conformational change in the AChE domain of neuroligin-2 to prevent dimerization and/or binding to neurexins. This type of secondary effect on neuroligin folding and thus dimerization/neurexin binding has been predicted for autism-associated mutations in neuroligins (Levinson and El-Husseini, 2007). For in-depth analysis of this type, crystallization of the MDGA1-neuroligin-2 complex would likely be required.

4.3.2.2 Analysis of MDGA Function *In Vivo*

An MDGA1 knockout mouse has previously been reported (Ishikawa et al., 2011). These mice displayed fairly normal overall brain morphology, but had an early

deficit in cortical migration. However, by P14, the deficit seems to be corrected and neurons appear to be in their proper positions (Ishikawa et al., 2011). This mouse could be used to examine the synaptic functions of MDGA1. Similar to the analysis briefly described for calsyntenins above, characterization could include examination of inhibitory and excitatory synapse density in brain slices and synaptic protein levels in subcellular fractions, as well as electrophysiological assessments. Given the recent links to schizophrenia and bipolar disorder for MDGA1 (Kahler et al., 2008; Li et al., 2011), it would be particularly important to include assays for schizophrenia-like symptoms, such as anxiety, anhedonia and impaired social behaviors, in a collection of behavioural tests for MDGA1 KO mice. Given the hypothesis that MDGA1 inhibits synaptic functions of neuroligin-2, an MDGA1 KO mouse may exhibit similar phenotypes to a neuroligin-2 overexpressing mouse. A neuroligin-2 overexpressing mouse has already been reported, and has increased inhibitory neurotransmission, a decreased E/I ratio, impaired social interactions, increased anxiety, stereotyped behaviours and increased seizures (Hines et al., 2008).

Alternatively, an MDGA1-overexpressing mouse could be generated. This type of genetic modification would be predicted to suppress neuroligin-2 function and might resemble a neuroligin-2 KO mouse phenotype. Neuroligin-2 KO mice have decreases in inhibitory neurotransmission and also show increases in anxiety, decreases in pain sensitivity and slight decreases in motor coordination (Blundell et al., 2009; Chubykin et al., 2007). Given that neuroligins, particularly neuroligin-1 and -2, play an important role in maintaining the E/I balance in the brain, suppression of neuroligin-2 through MDGA1 overexpression may also resemble a neuroligin-1

overexpressing mouse. Neuroligin-1 overexpressing mice show an increase in the E/I ratio, impairments in LTP and deficits in memory acquisition (Dahlhaus et al., 2010). Analysis of MDGA1 KO or overexpressing mice might also reveal other neuroligin-2-independent functions that are yet to be discovered.

Another intriguing, albeit more complex, possibility is using mouse models to investigate the interplay between MDGAs and neuroligins directly. For example, *in utereo* electroporation or crossing with an MDGA1-overexpressing mouse could be used to overexpress MDGA1 in a neuroligin-2 overexpressing mouse. Perhaps co-overexpression of MDGA1 could partially ameliorate the electrophysiological and behavioural phentotypes of neuroligin-2 overexpression. Alternatively, if MDGA1 KO has a synaptic effect, crossing with a neuroligin-2 KO could be attempted to determine if the synaptic effect is abolished in the absence of neuroligin-2. These types of approaches may be technically more challenging, but could prove that MDGAs play an important role in regulating neuroligin-2 at synapses, and thus in regulating the E/I balance in the brain.

4.3.2.3 The Synaptic Hypothesis of Neurodevelopmental Disorders

Since MDGA1 is linked to schizophrenia and bipolar disorder (Kahler et al., 2008; Li et al., 2011), and MDGA2 is linked to autism (Bucan et al., 2009), how these mutations may translate to a disease phenotype is another interesting area to pursue. This is technically difficult to accomplish for MDGA1, since the reported mutations are in intronic regions. However, MDGA2 mutations are exonic and could be modeled more readily. An initial test could be to clone MDGA2-associated mutations and compare binding to neuroligin-2. In Chapter 3, only neuroligin-2 co-

culture inhibition was reported for MDGA1; however, if MDGA2 can also inhibit neuroligin-2 in co-culture, additional tests are possible. It would be interesting to determine if a disease-associated MDGA2 mutant can also bind and inhibit neuroligin-2. If results look promising, this type of mutation could ultimately be modeled in a mouse where both synaptic function and behavior can be assessed, as has been done for other disease-associated mutations. In the case of the neuroligin-3 R451C mice, the behavioural and electrophysiological phenotype was not the same as that of a straight neuroligin-3 KO mouse, suggesting a gain-of-function effect (Tabuchi et al., 2007). Many of these types of model systems will have to be generated and analyzed in order to gain a full understanding of the complexities of neurodevelopmental disorders like autism and schizophrenia.

The fact that many genetic studies have linked synaptic proteins to ASDs and schizophrenia has led to a “synaptic” hypothesis for neurodevelopmental disorders (Abrahams and Geschwind, 2008; Betancur et al., 2009; Bourgeron, 2009; Sudhof, 2008; Zoghbi, 2003). This theory suggests that the disease phenotype may arise from mutation(s) in a wide range of “synaptic” proteins which would affect synapse formation and/or plasticity. The interplay between, and developmental timing of, synaptic deficits and other environmental or biological factors (such as epigenetics) would combine differently in every patient to produce the wide variety of clinical symptoms. Many of the ASD-linked genes have also been associated with schizophrenia, which suggests that these disorders may share underlying biological causes, and may not be clinically so distinct after all. In fact, some suggest that schizophrenia may belong on the autism spectrum, based on the possible common

biological underpinnings, as well as overlaps in clinical presentation, functional connectivity and the spectrum of symptoms seen in both disorders (King and Lord, 2011). It has also been suggested that ASDs may be considered “critical period” disorders, where problems with synaptic pruning or activity-dependent circuit refinement early in development may result in E/I disruptions and behavioural and cognitive alterations (LeBlanc and Fagiolini, 2011). Since synapses are plastic, this suggests that intervention during this critical period of development and synaptic refinement may be an effective treatment strategy. In the case of ASDs, onset occurs during the time of peak synapse formation and maturation, so a “synaptic” cause or risk factor is plausible. For schizophrenia however, onset occurs many years later; in this case perhaps just enough synapses are spared until additional pruning occurs during late-adolescence/early adulthood, when synaptic function may fall below a critical threshold, or synapses may simply be less stable later so over-pruning occurs (Faludi and Mirnics, 2011). Regardless, changes in synapse formation and stability could have a wide range of effects and would depend on temporal and spatial manifestation; such changes could represent a biological cause for autism or schizophrenia. Results from knockout and overexpressing mouse studies with synaptic proteins like neuroligins and neurexins show that even deleting a single cell adhesion molecule can have drastic effects on synaptic maturation, neurotransmission, E/I balances and behaviour.

However, it should be cautioned that interpretation of these genetic studies is not clear-cut. As in the case of MDGA1, many of the reported mutations in synaptic proteins are in intronic regions and therefore do not code for any part of the

translated protein; as such, it is difficult to predict how these genetic changes may translate into functional changes at synapses. In addition, penetrance for mutations seems to be imperfect, with reports of asymptomatic family members carrying the mutation, family members carrying the mutation with a different clinical presentation (i.e. autism vs. schizophrenia), as well as the occurrence of affected/symptomatic family members who do not carry the presumed pathogenic variant (Mitchell, 2011). Therefore, it seems likely that in a given affected individual, the genetic mutation is influenced by other independently segregated genetic factors and/or environmental influences that interact to result in a clinical phenotype. Further study is clearly needed to determine how so many synapse-associated mutations, converging on synaptic function, can result in the varying degrees of behavioural and cognitive symptoms of neurodevelopmental disorders.

4.4 Concluding Remarks

The work presented here identifies two new synaptic organizing proteins: calsyntenin-3 and MDGAs. It was shown that these proteins are both related to the well-characterized neurexin-neuroligin pair: postsynaptic calsyntenin-3 directly binds presynaptic neurexin-1α and induces excitatory and inhibitory presynaptic differentiation, while postsynaptic MDGAs bind postsynaptic neuroligin-2 to specifically decrease inhibitory synapse development. These findings thus represent two new modes by which synaptic development can be regulated, and place calsyntenin-3 and MDGAs in league with a myriad of other postsynaptic synaptic cell adhesion molecules such as LRRTMs, SynCAMs, ephrins/Eph receptors, SALMs,

APP, NGLs, TrkC and Slitrk3 (Siddiqui and Craig, 2011). The fact that there are so many proteins able to induce synaptic differentiation supports the idea that synaptogenesis is a very important biological process. Numerous molecular players may allow for redundancy or the ability to compensate, and they likely also add mechanical stability between pre- and postsynaptic sides. Culture studies show that not all aspects of synapse formation or maturation can be mediated by a single molecule, so it is likely that different protein families cooperate *in vivo*. A number of different postsynaptic adhesion molecules, many of which have known intracellular binding partners, may also aid in the nucleation of the dense meshwork of proteins in the postsynaptic compartment that allow for the rapid response to neurotransmitter release, as well as the longer-term signaling capabilities that are needed to mediate synaptic plasticity; some proteins are also directly involved in mediating plasticity via signaling with intracellular partners. Last, the variety of pre- and postsynaptic molecules may account for synaptic specificity, determining which partners will form synaptic connections and even controlling subcellular placement of synapses.

Despite the ever-growing number of synaptic cell adhesion molecules, neurexins, with their multiple isoforms, splice variants, and binding partners, are still the best candidates as organizers of synaptogenesis in the CNS. In this study, calsyntenin-3 is shown to be a new binding partner for neurexin. Further characterization of the varying binding affinities and expression patterns of neurexin isoforms to different postsynaptic partners will also shed light on how these presynaptic proteins may instruct synaptic development. It will be interesting to

follow-up the initial binding results with *in vivo* analysis of calsyntenin-3 function and compare this to known *in vivo* roles for neurexins. Calsyntenin-3 may also exhibit unique regulatory mechanisms due to extracellular proteolytic processing, which will also be interesting to further investigate.

Neuroligin-2, the main inhibitory postsynaptic CAM, has a well-established role in inhibitory synapse maturation and function. MDGAs, via direct binding to neuroligin-2 and blocking bi-directional signaling with neurexin, may therefore play a critical role in inhibitory synapse development. The function of MDGAs as negative regulators of neuroligin-2 represents a new mode for controlling the actions of neuroligin-2, and is likely involved in maintaining the correct E/I balance in the brain. Such a balance is critical for cognitive function and behavior, and disruptions in this balance may lead to behavioural or cognitive malfunctions, such as those seen in neurodevelopmental disorders. Further analysis of the *in vivo* interplay between MDGAs and neuroligin-2 will help determine how MDGAs influence inhibitory synapse development.

Synapses are the basic units of communication in the brain. Understanding how they form, mature, and change in response to activity and the environment is essential to understanding how the brain functions as a whole, and ultimately, how complex behaviors are produced. A comprehensive grasp of how synapses function under healthy conditions is also critical to determine how synaptic function may be altered in disease - from neurodevelopmental disorders like autism to neurodegenerative disorders like Alzheimer's - and will hopefully lead to the eventual development of new therapeutic approaches.

References

Abrahams, B.S., and Geschwind, D.H. (2008). Advances in autism genetics: on the threshold of a new neurobiology. Nat Rev Genet *9*, 341-355.

Ahmari, S.E., Buchanan, J., and Smith, S.J. (2000). Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci *3*, 445-451.

Aiga, M., Levinson, J.N., and Bamji, S.X. (2011). N-cadherin and neuroligins cooperate to regulate synapse formation in hippocampal cultures. J Biol Chem *286*, 851-858.

Akert, K. (1971). Fine structure of neurons and synapses in the central nervous system. Nord Med *86*, 1219.

Akins, M.R., and Biederer, T. (2006). Cell-cell interactions in synaptogenesis. Curr Opin Neurobiol *16*, 83-89.

Aldinger, K.A., Plummer, J.T., Qiu, S., and Levitt, P. (2011). SnapShot: genetics of autism. Neuron *72*, 418-418 e411.

Alvarez, V.A., Ridenour, D.A., and Sabatini, B.L. (2006). Retraction of synapses and dendritic spines induced by off-target effects of RNA interference. J Neurosci *26*, 7820-7825.

American Psychiatric Association., and American Psychiatric Association. Task Force on DSM-IV. (2000). Diagnostic and statistical manual of mental disorders : DSM-IV-TR, 4th edn (Washington, DC, American Psychiatric Association).

Amir, R.E., Van den Veyver, I.B., Wan, M., Tran, C.Q., Francke, U., and Zoghbi, H.Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nature genetics *23*, 185-188.

Andersen, P. (2007). The hippocampus book (Oxford ; New York, Oxford University Press).

Aoto, J., Ting, P., Maghsoodi, B., Xu, N., Henkemeyer, M., and Chen, L. (2007). Postsynaptic ephrinB3 promotes shaft glutamatergic synapse formation. J Neurosci *27*, 7508-7519.

Arac, D., Boucard, A.A., Ozkan, E., Strop, P., Newell, E., Sudhof, T.C., and Brunger, A.T. (2007). Structures of neuroligin-1 and the neuroligin-1/neurexin-1 beta complex reveal specific protein-protein and protein-Ca2+ interactions. Neuron *56*, 992-1003.

Araki, Y., Kawano, T., Taru, H., Saito, Y., Wada, S., Miyamoto, K., Kobayashi, H., Ishikawa, H.O., Ohsugi, Y., Yamamoto, T.*, et al.* (2007). The novel cargo Alcadein induces vesicle association of kinesin-1 motor components and activates axonal transport. Embo J *26*, 1475-1486.

Araki, Y., Miyagi, N., Kato, N., Yoshida, T., Wada, S., Nishimura, M., Komano, H., Yamamoto, T., De Strooper, B., Yamamoto, K.*, et al.* (2004). Coordinated metabolism of Alcadein and amyloid beta-protein precursor regulates FE65-dependent gene transactivation. J Biol Chem *279*, 24343-24354.

Araki, Y., Tomita, S., Yamaguchi, H., Miyagi, N., Sumioka, A., Kirino, Y., and Suzuki, T. (2003). Novel cadherin-related membrane proteins, Alcadeins, enhance the X11-like protein-mediated stabilization of amyloid beta-protein precursor metabolism. J Biol Chem *278*, 49448-49458.

Arikkath, J., and Reichardt, L.F. (2008). Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. Trends Neurosci *31*, 487-494.

Bakkaloglu, B., O'Roak, B.J., Louvi, A., Gupta, A.R., Abelson, J.F., Morgan, T.M., Chawarska, K., Klin, A., Ercan-Sencicek, A.G., Stillman, A.A.*, et al.* (2008). Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. Am J Hum Genet *82*, 165-173.

Bamji, S.X., Shimazu, K., Kimes, N., Huelsken, J., Birchmeier, W., Lu, B., and Reichardt, L.F. (2003). Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. Neuron *40*, 719-731.

Banker, G., and Goslin, K. (1998). Culturing nerve cells, 2nd edn (Cambridge, Mass., MIT Press).

Banker, G.A., and Cowan, W.M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res *126*, 397-342.

Bartlett, W.P., and Banker, G.A. (1984). An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. II. Synaptic relationships. J Neurosci *4*, 1954-1965.

Bear, M.F., Huber, K.M., and Warren, S.T. (2004). The mGluR theory of fragile X mental retardation. Trends Neurosci *27*, 370-377.

Beglopoulos, V., Montag-Sallaz, M., Rohlmann, A., Piechotta, K., Ahmad, M., Montag, D., and Missler, M. (2005). Neurexophilin 3 is highly localized in cortical and cerebellar regions and is functionally important for sensorimotor gating and motor coordination. Mol Cell Biol *25*, 7278-7288.

Benson, D.L., Watkins, F.H., Steward, O., and Banker, G. (1994). Characterization of GABAergic neurons in hippocampal cell cultures. J Neurocytol *23*, 279-295.

Berkel, S., Marshall, C.R., Weiss, B., Howe, J., Roeth, R., Moog, U., Endris, V., Roberts, W., Szatmari, P., Pinto, D.*, et al.* (2010). Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. Nature genetics *42*, 489-491.

Betancur, C., Sakurai, T., and Buxbaum, J.D. (2009). The emerging role of synaptic cell-adhesion pathways in the pathogenesis of autism spectrum disorders. Trends Neurosci *32*, 402-412.

Bhalla, K., Luo, Y., Buchan, T., Beachem, M.A., Guzauskas, G.F., Ladd, S., Bratcher, S.J., Schroer, R.J., Balsamo, J., DuPont, B.R.*, et al.* (2008). Alterations in CDH15 and KIRREL3 in patients with mild to severe intellectual disability. Am J Hum Genet *83*, 703-713.

Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E.T., and Sudhof, T.C. (2002). SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science *297*, 1525-1531.

Biederer, T., and Sudhof, T.C. (2000). Mints as adaptors. Direct binding to neurexins and recruitment of munc18. J Biol Chem *275*, 39803-39806.

Biederer, T., and Sudhof, T.C. (2001). CASK and protein 4.1 support F-actin nucleation on neurexins. J Biol Chem *276*, 47869-47876.

Birks, R., Huxley, H.E., and Katz, B. (1960). The fine structure of the neuromuscular junction of the frog. J Physiol *150*, 134-144.

Black, R.A., Doedens, J.R., Mahimkar, R., Johnson, R., Guo, L., Wallace, A., Virca, D., Eisenman, J., Slack, J., Castner, B.*, et al.* (2003). Substrate specificity and inducibility of TACE (tumour necrosis factor alpha-converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity. Biochem Soc Symp, 39-52.

Blundell, J., Blaiss, C.A., Etherton, M.R., Espinosa, F., Tabuchi, K., Walz, C., Bolliger, M.F., Sudhof, T.C., and Powell, C.M. (2010). Neuroligin-1 deletion results in impaired spatial memory and increased repetitive behavior. J Neurosci *30*, 2115-2129.

Blundell, J., Tabuchi, K., Bolliger, M.F., Blaiss, C.A., Brose, N., Liu, X., Sudhof, T.C., and Powell, C.M. (2009). Increased anxiety-like behavior in mice lacking the inhibitory synapse cell adhesion molecule neuroligin 2. Genes Brain Behav *8*, 114-126.

Bolliger, M.F., Frei, K., Winterhalter, K.H., and Gloor, S.M. (2001). Identification of a novel neuroligin in humans which binds to PSD-95 and has a widespread expression. Biochem J *356*, 581-588.

Bolshakov, V.Y., and Siegelbaum, S.A. (1995). Regulation of hippocampal transmitter release during development and long-term potentiation. Science *269*, 1730-1734.

Bonaglia, M.C., Giorda, R., Mani, E., Aceti, G., Anderlid, B.M., Baroncini, A., Pramparo, T., and Zuffardi, O. (2006). Identification of a recurrent breakpoint within the SHANK3 gene in the 22q13.3 deletion syndrome. J Med Genet *43*, 822-828.

Boucard, A.A., Chubykin, A.A., Comoletti, D., Taylor, P., and Sudhof, T.C. (2005). A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. Neuron *48*, 229-236.

Boucard, A.A., Ko, J., and Sudhof, T.C. (2012). High-affinity neurexin binding to the cell-adhesion G-protein coupled receptor CIRL1/Latrophilin-1 produces an intercellular adhesion complex. J Biol Chem.

Bourgeron, T. (2009). A synaptic trek to autism. Curr Opin Neurobiol *19*, 231-234.

Bourne, J., and Harris, K.M. (2007). Do thin spines learn to be mushroom spines that remember? Curr Opin Neurobiol *17*, 381-386.

Bozdagi, O., Sakurai, T., Papapetrou, D., Wang, X., Dickstein, D.L., Takahashi, N., Kajiwara, Y., Yang, M., Katz, A.M., Scattoni, M.L.*, et al.* (2010). Haploinsufficiency of the autism-associated Shank3 gene leads to deficits in synaptic function, social interaction, and social communication. Mol Autism *1*, 15.

Bredt, D.S., and Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. Neuron *40*, 361-379.

Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L., and Iggo, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet *34*, 263-264.

Brigidi, G.S., and Bamji, S.X. (2011). Cadherin-catenin adhesion complexes at the synapse. Curr Opin Neurobiol *21*, 208-214.

Brose, K., and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol *10*, 95-102.

Bucan, M., Abrahams, B.S., Wang, K., Glessner, J.T., Herman, E.I., Sonnenblick, L.I., Alvarez Retuerto, A.I., Imielinski, M., Hadley, D., Bradfield, J.P.*, et al.* (2009). Genome-wide analyses of exonic copy number variants in a family-based study point to novel autism susceptibility genes. PLoS Genet *5*, e1000536.

Budreck, E.C., and Scheiffele, P. (2007). Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. Eur J Neurosci *26*, 1738-1748.

Burns, M.E., and Augustine, G.J. (1995). Synaptic structure and function: dynamic organization yields architectural precision. Cell *83*, 187-194.

Bury, L.A., and Sabo, S.L. (2010). How it's made: the synapse. Mol Interv *10*, 282-292.

Butz, S., Okamoto, M., and Sudhof, T.C. (1998). A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. Cell *94*, 773-782.

Canitano, R. (2007). Epilepsy in autism spectrum disorders. Eur Child Adolesc Psychiatry *16*, 61-66.

Carninci, P., Kvam, C., Kitamura, A., Ohsumi, T., Okazaki, Y., Itoh, M., Kamiya, M., Shibata, K., Sasaki, N., Izawa, M.*, et al.* (1996). High-efficiency full-length cDNA cloning by biotinylated CAP trapper. Genomics *37*, 327-336.

Cerpa, W., Godoy, J.A., Alfaro, I., Farias, G.G., Metcalfe, M.J., Fuentealba, R., Bonansco, C., and Inestrosa, N.C. (2008). Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. J Biol Chem *283*, 5918-5927.

Chadman, K.K., Gong, S., Scattoni, M.L., Boltuck, S.E., Gandhy, S.U., Heintz, N., and Crawley, J.N. (2008). Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice. Autism Res *1*, 147-158.

Charron, F., and Tessier-Lavigne, M. (2005). Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development *132*, 2251-2262.

Chavis, P., and Westbrook, G. (2001). Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. Nature *411*, 317-321.

Chen, F., Venugopal, V., Murray, B., and Rudenko, G. (2011). The structure of neurexin 1alpha reveals features promoting a role as synaptic organizer. Structure *19*, 779-789.

Chen, J.L., and Nedivi, E. (2010). Neuronal structural remodeling: is it all about access? Curr Opin Neurobiol *20*, 557-562.

Chen, L., Chetkovich, D.M., Petralia, R.S., Sweeney, N.T., Kawasaki, Y., Wenthold, R.J., Bredt, D.S., and Nicoll, R.A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature *408*, 936-943.

Chen, R.Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nature genetics *27*, 327-331.

Chen, X., Liu, H., Shim, A.H., Focia, P.J., and He, X. (2008). Structural basis for synaptic adhesion mediated by neuroligin-neurexin interactions. Nat Struct Mol Biol *15*, 50-56.

Chih, B., Afridi, S.K., Clark, L., and Scheiffele, P. (2004). Disorder-associated mutations lead to functional inactivation of neuroligins. Hum Mol Genet *13*, 1471-1477.

Chih, B., Engelman, H., and Scheiffele, P. (2005). Control of excitatory and inhibitory synapse formation by neuroligins. Science *307*, 1324-1328.

Chih, B., Gollan, L., and Scheiffele, P. (2006). Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. Neuron *51*, 171-178.

Cho, R.W., Park, J.M., Wolff, S.B., Xu, D., Hopf, C., Kim, J.A., Reddy, R.C., Petralia, R.S., Perin, M.S., Linden, D.J.*, et al.* (2008). mGluR1/5-dependent long-term depression requires the regulated ectodomain cleavage of neuronal pentraxin NPR by TACE. Neuron *57*, 858-871.

Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., and Barres, B.A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell *120*, 421-433.

Chubykin, A.A. (2009). Neurexins and Neuroligins: A Synaptic Code for Neuronal Wiring That Is Implicated in Autism. 347-365.

Chubykin, A.A., Atasoy, D., Etherton, M.R., Brose, N., Kavalali, E.T., Gibson, J.R., and Sudhof, T.C. (2007). Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. Neuron *54*, 919-931.

Clayton-Smith, J., and Laan, L. (2003). Angelman syndrome: a review of the clinical and genetic aspects. J Med Genet *40*, 87-95.

Colonnier, M. (1968). Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. Brain Res *9*, 268-287.

Comoletti, D., De Jaco, A., Jennings, L.L., Flynn, R.E., Gaietta, G., Tsigelny, I., Ellisman, M.H., and Taylor, P. (2004). The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. J Neurosci *24*, 4889-4893.

Comoletti, D., Flynn, R., Jennings, L.L., Chubykin, A., Matsumura, T., Hasegawa, H., Sudhof, T.C., and Taylor, P. (2003). Characterization of the interaction of a recombinant soluble neuroligin-1 with neurexin-1beta. J Biol Chem *278*, 50497-50505.

Comoletti, D., Flynn, R.E., Boucard, A.A., Demeler, B., Schirf, V., Shi, J., Jennings, L.L., Newlin, H.R., Sudhof, T.C., and Taylor, P. (2006). Gene selection, alternative splicing, and post-translational processing regulate neuroligin selectivity for beta-neurexins. Biochemistry *45*, 12816-12827.

Cook, E.H., Jr., and Scherer, S.W. (2008). Copy-number variations associated with neuropsychiatric conditions. Nature *455*, 919-923.

Courchesne, E., Karns, C.M., Davis, H.R., Ziccardi, R., Carper, R.A., Tigue, Z.D., Chisum, H.J., Moses, P., Pierce, K., Lord, C.*, et al.* (2001). Unusual brain growth patterns in early life in patients with autistic disorder: an MRI study. Neurology *57*, 245-254.

Courchesne, E., Pierce, K., Schumann, C.M., Redcay, E., Buckwalter, J.A., Kennedy, D.P., and Morgan, J. (2007). Mapping early brain development in autism. Neuron *56*, 399-413.

Couteaux, R., and Pecot-Dechavassine, M. (1970). [Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction]. C R Acad Sci Hebd Seances Acad Sci D *271*, 2346-2349.

Cowan, W.M., Sudhof, T.C., Stevens, C.F., and Davies, K. (2001). Synapses (Baltimore, Md. ; London, Johns Hopkins University Press).

Craig, A.M., and Boudin, H. (2001). Molecular heterogeneity of central synapses: afferent and target regulation. Nat Neurosci *4*, 569-578.

Craig, A.M., Graf, E.R., and Linhoff, M.W. (2006). How to build a central synapse: clues from cell culture. Trends Neurosci *29*, 8-20.

Craig, A.M., and Kang, Y. (2007). Neurexin-neuroligin signaling in synapse development. Curr Opin Neurobiol *17*, 43-52.

Cusmano-Ozog, K., Manning, M.A., and Hoyme, H.E. (2007). 22q13.3 deletion syndrome: a recognizable malformation syndrome associated with marked speech and language delay. Am J Med Genet C Semin Med Genet *145C*, 393-398.

Dahlhaus, R., Hines, R.M., Eadie, B.D., Kannangara, T.S., Hines, D.J., Brown, C.E., Christie, B.R., and El-Husseini, A. (2010). Overexpression of the cell adhesion protein neuroligin-1 induces learning deficits and impairs synaptic plasticity by altering the ratio of excitation to inhibition in the hippocampus. Hippocampus *20*, 305-322.

Dalva, M.B., McClelland, A.C., and Kayser, M.S. (2007). Cell adhesion molecules: signalling functions at the synapse. Nat Rev Neurosci *8*, 206-220.

Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W., and Greenberg, M.E. (2000). EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell *103*, 945-956.

Davies, M., Bateson, A.N., and Dunn, S.M. (1996). Molecular biology of the GABA(A) receptor: functional domains implicated by mutational analysis. Front Biosci *1*, d214-233.

de Wit, J., Sylwestrak, E., O'Sullivan, M.L., Otto, S., Tiglio, K., Savas, J.N., Yates, J.R., 3rd, Comoletti, D., Taylor, P., and Ghosh, A. (2009). LRRTM2 interacts with Neurexin1 and regulates excitatory synapse formation. Neuron *64*, 799-806.

Dean, C., and Dresbach, T. (2006). Neuroligins and neurexins: linking cell adhesion, synapse formation and cognitive function. Trends Neurosci *29*, 21-29.

Dean, C., Scholl, F.G., Choih, J., DeMaria, S., Berger, J., Isacoff, E., and Scheiffele, P. (2003). Neurexin mediates the assembly of presynaptic terminals. Nat Neurosci *6*, 708-716.

DeLorey, T.M., Handforth, A., Anagnostaras, S.G., Homanics, G.E., Minassian, B.A., Asatourian, A., Fanselow, M.S., Delgado-Escueta, A., Ellison, G.D., and Olsen, R.W. (1998). Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. J Neurosci *18*, 8505-8514.

DeLorey, T.M., Sahbaie, P., Hashemi, E., Homanics, G.E., and Clark, J.D. (2008). Gabrb3 gene deficient mice exhibit impaired social and exploratory behaviors, deficits in non-selective attention and hypoplasia of cerebellar vermal lobules: a potential model of autism spectrum disorder. Behav Brain Res *187*, 207-220.

Diaz-Lopez, A., Rivas, C., Iniesta, P., Moran, A., Garcia-Aranda, C., Megias, D., Sanchez-Pernaute, A., Torres, A., Diaz-Rubio, E., Benito, M.*, et al.* (2005). Characterization of MDGA1, a novel human glycosylphosphatidylinositol-anchored protein localized in lipid rafts. Exp Cell Res *307*, 91-99.

Dityatev, A., and El-Husseini, A. (2006). Molecular mechanisms of synaptogenesis (New York, Springer Science+Business Media).

Dresbach, T., Neeb, A., Meyer, G., Gundelfinger, E.D., and Brose, N. (2004). Synaptic targeting of neuroligin is independent of neurexin and SAP90/PSD95 binding. Mol Cell Neurosci *27*, 227-235.

Dudanova, I., Tabuchi, K., Rohlmann, A., Sudhof, T.C., and Missler, M. (2007). Deletion of alpha-neurexins does not cause a major impairment of axonal pathfinding or synapse formation. J Comp Neurol *502*, 261-274.

Durand, C.M., Betancur, C., Boeckers, T.M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I.C., Anckarsater, H.*, et al.* (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nature genetics *39*, 25-27.

Durand, G.M., Kovalchuk, Y., and Konnerth, A. (1996). Long-term potentiation and functional synapse induction in developing hippocampus. Nature *381*, 71-75.

Ehlers, M.D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat Neurosci *6*, 231-242.

Ehrlich, I., Klein, M., Rumpel, S., and Malinow, R. (2007). PSD-95 is required for activity-driven synapse stabilization. Proc Natl Acad Sci U S A *104*, 4176-4181.

Elia, L.P., Yamamoto, M., Zang, K., and Reichardt, L.F. (2006). p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. Neuron *51*, 43-56.

Eroglu, C., Allen, N.J., Susman, M.W., O'Rourke, N.A., Park, C.Y., Ozkan, E., Chakraborty, C., Mulinyawe, S.B., Annis, D.S., Huberman, A.D.*, et al.* (2009). Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell *139*, 380-392.

Ethell, I.M., and Ethell, D.W. (2007). Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. J Neurosci Res *85*, 2813-2823.

Etherton, M., Foldy, C., Sharma, M., Tabuchi, K., Liu, X., Shamloo, M., Malenka, R.C., and Sudhof, T.C. (2011). Autism-linked neuroligin-3 R451C mutation differentially alters hippocampal and cortical synaptic function. Proc Natl Acad Sci U S A *108*, 13764-13769.

Ey, E., Leblond, C.S., and Bourgeron, T. (2011). Behavioral profiles of mouse models for autism spectrum disorders. Autism Res *4*, 5-16.

Fabrichny, I.P., Leone, P., Sulzenbacher, G., Comoletti, D., Miller, M.T., Taylor, P., Bourne, Y., and Marchot, P. (2007). Structural analysis of the synaptic protein neuroligin and its beta-neurexin complex: determinants for folding and cell adhesion. Neuron *56*, 979-991.

Fallin, M.D., Lasseter, V.K., Avramopoulos, D., Nicodemus, K.K., Wolyniec, P.S., McGrath, J.A., Steel, G., Nestadt, G., Liang, K.Y., Huganir, R.L.*, et al.* (2005). Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. Am J Hum Genet *77*, 918-936.

Faludi, G., and Mirnics, K. (2011). Synaptic changes in the brain of subjects with schizophrenia. Int J Dev Neurosci *29*, 305-309.

Fannon, A.M., and Colman, D.R. (1996). A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins. Neuron *17*, 423-434.

Feng, J., Schroer, R., Yan, J., Song, W., Yang, C., Bockholt, A., Cook, E.H., Jr., Skinner, C., Schwartz, C.E., and Sommer, S.S. (2006). High frequency of neurexin 1beta signal peptide structural variants in patients with autism. Neurosci Lett *409*, 10-13.

Fernell, E., and Gillberg, C. (2010). Autism spectrum disorder diagnoses in Stockholm preschoolers. Res Dev Disabil *31*, 680-685.

Fletcher, T.L., De Camilli, P., and Banker, G. (1994). Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. J Neurosci *14*, 6695-6706.

Fogel, A.I., Akins, M.R., Krupp, A.J., Stagi, M., Stein, V., and Biederer, T. (2007). SynCAMs organize synapses through heterophilic adhesion. J Neurosci *27*, 12516-12530.

Francks, C., Maegawa, S., Lauren, J., Abrahams, B.S., Velayos-Baeza, A., Medland, S.E., Colella, S., Groszer, M., McAuley, E.Z., Caffrey, T.M.*, et al.* (2007). LRRTM1 on chromosome 2p12 is a maternally suppressed gene that is associated paternally with handedness and schizophrenia. Mol Psychiatry *12*, 1129-1139, 1057.

Froyen, G., Van Esch, H., Bauters, M., Hollanders, K., Frints, S.G., Vermeesch, J.R., Devriendt, K., Fryns, J.P., and Marynen, P. (2007). Detection of genomic copy number changes in patients with idiopathic mental retardation by high-resolution X-array-CGH: important role for increased gene dosage of XLMR genes. Hum Mutat *28*, 1034-1042.

Fu, M., and Zuo, Y. (2011). Experience-dependent structural plasticity in the cortex. Trends Neurosci *34*, 177-187.

Fujimura, Y., Iwashita, M., Matsuzaki, F., and Yamamoto, T. (2006). MDGA1, an IgSF molecule containing a MAM domain, heterophilically associates with axon- and muscle-associated binding partners through distinct structural domains. Brain Res *1101*, 12-19.

Futai, K., Kim, M.J., Hashikawa, T., Scheiffele, P., Sheng, M., and Hayashi, Y. (2007). Retrograde modulation of presynaptic release probability through signaling mediated by PSD-95-neuroligin. Nat Neurosci *10*, 186-195.

Garber, K.B., Visootsak, J., and Warren, S.T. (2008). Fragile X syndrome. Eur J Hum Genet *16*, 666-672.

Garner, C.C., Zhai, R.G., Gundelfinger, E.D., and Ziv, N.E. (2002). Molecular mechanisms of CNS synaptogenesis. Trends Neurosci *25*, 243-251.

Gauthier, J., Champagne, N., Lafreniere, R.G., Xiong, L., Spiegelman, D., Brustein, E., Lapointe, M., Peng, H., Cote, M., Noreau, A.*, et al.* (2010). De novo mutations in the gene encoding the synaptic scaffolding protein SHANK3 in patients ascertained for schizophrenia. Proc Natl Acad Sci U S A *107*, 7863-7868.

Gauthier, J., Siddiqui, T.J., Huashan, P., Yokomaku, D., Hamdan, F.F., Champagne, N., Lapointe, M., Spiegelman, D., Noreau, A., Lafreniere, R.G.*, et al.* (2011). Truncating mutations in NRXN2 and NRXN1 in autism spectrum disorders and schizophrenia. Hum Genet *130*, 563-573.

Gibson, J.R., Huber, K.M., and Sudhof, T.C. (2009). Neuroligin-2 deletion selectively decreases inhibitory synaptic transmission originating from fast-spiking but not from somatostatin-positive interneurons. J Neurosci *29*, 13883-13897.

Gillberg, C., and Billstedt, E. (2000). Autism and Asperger syndrome: coexistence with other clinical disorders. Acta Psychiatr Scand *102*, 321-330.

Glessner, J.T., Wang, K., Cai, G., Korvatska, O., Kim, C.E., Wood, S., Zhang, H., Estes, A., Brune, C.W., Bradfield, J.P.*, et al.* (2009). Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. Nature *459*, 569-573.

Gogolla, N., Galimberti, I., Deguchi, Y., and Caroni, P. (2009). Wnt signaling mediates experience-related regulation of synapse numbers and mossy fiber connectivities in the adult hippocampus. Neuron *62*, 510-525.

Grady, R.M., Zhou, H., Cunningham, J.M., Henry, M.D., Campbell, K.P., and Sanes, J.R. (2000). Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin--glycoprotein complex. Neuron *25*, 279-293.

Graf, E.R., Kang, Y., Hauner, A.M., and Craig, A.M. (2006). Structure function and splice site analysis of the synaptogenic activity of the neurexin-1 beta LNS domain. J Neurosci *26*, 4256-4265.

Graf, E.R., Zhang, X., Jin, S.X., Linhoff, M.W., and Craig, A.M. (2004). Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell *119*, 1013-1026.

Gray, E.G. (1959). Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. Nature *183*, 1592-1593.

Gray, E.G. (1963). Electron microscopy of presynaptic organelles of the spinal cord. J Anat *97*, 101-106.

Greer, P.L., and Greenberg, M.E. (2008). From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron *59*, 846-860.

Greger, I.H., Ziff, E.B., and Penn, A.C. (2007). Molecular determinants of AMPA receptor subunit assembly. Trends Neurosci *30*, 407-416.

Grootjans, J.J., Reekmans, G., Ceulemans, H., and David, G. (2000). Syntenin-syndecan binding requires syndecan-synteny and the co-operation of both PDZ domains of syntenin. J Biol Chem *275*, 19933-19941.

Guilmatre, A., Dubourg, C., Mosca, A.L., Legallic, S., Goldenberg, A., Drouin-Garraud, V., Layet, V., Rosier, A., Briault, S., Bonnet-Brilhault, F.*, et al.* (2009). Recurrent rearrangements in synaptic and neurodevelopmental genes and shared biologic pathways in schizophrenia, autism, and mental retardation. Arch Gen Psychiatry *66*, 947-956.

Guy, J., Gan, J., Selfridge, J., Cobb, S., and Bird, A. (2007). Reversal of neurological defects in a mouse model of Rett syndrome. Science *315*, 1143-1147.

Harris, K.M., and Stevens, J.K. (1989). Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. J Neurosci *9*, 2982-2997.

Hata, S., Fujishige, S., Araki, Y., Kato, N., Araseki, M., Nishimura, M., Hartmann, D., Saftig, P., Fahrenholz, F., Taniguchi, M.*, et al.* (2009). Alcadein cleavages by amyloid beta-precursor protein (APP) alpha- and gamma-secretases generate small peptides, p3-Alcs, indicating Alzheimer disease-related gamma-secretase dysfunction. J Biol Chem *284*, 36024-36033.

Hata, Y., Butz, S., and Sudhof, T.C. (1996). CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. J Neurosci *16*, 2488-2494.

Hata, Y., Davletov, B., Petrenko, A.G., Jahn, R., and Sudhof, T.C. (1993). Interaction of synaptotagmin with the cytoplasmic domains of neurexins. Neuron *10*, 307-315.

Henderson, J.T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder, J.C., and Pawson, T. (2001). The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. Neuron *32*, 1041-1056.

Henkemeyer, M., Itkis, O.S., Ngo, M., Hickmott, P.W., and Ethell, I.M. (2003). Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. J Cell Biol *163*, 1313-1326.

Hines, R.M., Wu, L., Hines, D.J., Steenland, H., Mansour, S., Dahlhaus, R., Singaraja, R.R., Cao, X., Sammler, E., Hormuzdi, S.G.*, et al.* (2008). Synaptic imbalance, stereotypies, and impaired social interactions in mice with altered neuroligin 2 expression. J Neurosci *28*, 6055-6067.

Hinton, V.J., Brown, W.T., Wisniewski, K., and Rudelli, R.D. (1991). Analysis of neocortex in three males with the fragile X syndrome. Am J Med Genet *41*, 289-294.

Hintsch, G., Zurlinden, A., Meskenaite, V., Steuble, M., Fink-Widmer, K., Kinter, J., and Sonderegger, P. (2002). The Calsyntenins—A Family of Postsynaptic Membrane Proteins with Distinct Neuronal Expression Patterns. Molecular and Cellular Neuroscience *21*, 393-409.

Hirai, H., Pang, Z., Bao, D., Miyazaki, T., Li, L., Miura, E., Parris, J., Rong, Y., Watanabe, M., Yuzaki, M.*, et al.* (2005). Cbln1 is essential for synaptic integrity and plasticity in the cerebellum. Nat Neurosci *8*, 1534-1541.

Hirano, S., Wang, X., and Suzuki, S.T. (2002). Restricted expression of protocadherin 2A in the developing mouse brain. Brain Res Mol Brain Res *98*, 119-123.

Hirao, K., Hata, Y., Ide, N., Takeuchi, M., Irie, M., Yao, I., Deguchi, M., Toyoda, A., Sudhof, T.C., and Takai, Y. (1998). A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. J Biol Chem *273*, 21105-21110.

Hirokawa, N., Pfister, K.K., Yorifuji, H., Wagner, M.C., Brady, S.T., and Bloom, G.S. (1989a). Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. Cell *56*, 867-878.

Hirokawa, N., Sobue, K., Kanda, K., Harada, A., and Yorifuji, H. (1989b). The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin 1. J Cell Biol *108*, 111-126.

Hoerndli, F.J., Walser, M., Frohli Hoier, E., de Quervain, D., Papassotiropoulos, A., and Hajnal, A. (2009). A conserved function of C. elegans CASY-1 calsyntenin in associative learning. PLoS One *4*, e4880.

Homanics, G.E., DeLorey, T.M., Firestone, L.L., Quinlan, J.J., Handforth, A., Harrison, N.L., Krasowski, M.D., Rick, C.E., Korpi, E.R., Makela, R.*, et al.* (1997). Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A *94*, 4143-4148.

Hoon, M., Soykan, T., Falkenburger, B., Hammer, M., Patrizi, A., Schmidt, K.F., Sassoe-Pognetto, M., Lowel, S., Moser, T., Taschenberger, H.*, et al.* (2011). Neuroligin-4 is localized to glycinergic postsynapses and regulates inhibition in the retina. Proc Natl Acad Sci U S A *108*, 3053-3058.

Huang, Y.Y., Bach, M.E., Lipp, H.P., Zhuo, M., Wolfer, D.P., Hawkins, R.D., Schoonjans, L., Kandel, E.R., Godfraind, J.M., Mulligan, R.*, et al.* (1996). Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways. Proc Natl Acad Sci U S A *93*, 8699-8704.

Huovila, A.P., Turner, A.J., Pelto-Huikko, M., Karkkainen, I., and Ortiz, R.M. (2005). Shedding light on ADAM metalloproteinases. Trends Biochem Sci *30*, 413-422.

Huttenlocher, P.R., and Dabholkar, A.S. (1997). Regional differences in synaptogenesis in human cerebral cortex. J Comp Neurol *387*, 167-178.

Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Sudhof, T.C. (1995). Neuroligin 1: a splice site-specific ligand for beta-neurexins. Cell *81*, 435-443.

Ichtchenko, K., Nguyen, T., and Sudhof, T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. J Biol Chem *271*, 2676-2682.

Ide, N., Hata, Y., Deguchi, M., Hirao, K., Yao, I., and Takai, Y. (1999). Interaction of S-SCAM with neural plakophilin-related Armadillo-repeat protein/delta-catenin. Biochem Biophys Res Commun *256*, 456-461.

Iijima, T., Wu, K., Witte, H., Hanno-Iijima, Y., Glatter, T., Richard, S., and Scheiffele, P. (2011). SAM68 regulates neuronal activity-dependent alternative splicing of neurexin-1. Cell *147*, 1601-1614.

Ikeda, D.D., Duan, Y., Matsuki, M., Kunitomo, H., Hutter, H., Hedgecock, E.M., and Iino, Y. (2008). CASY-1, an ortholog of calsyntenins/alcadeins, is essential for learning in Caenorhabditis elegans. Proc Natl Acad Sci U S A *105*, 5260-5265.

Inestrosa, N.C., and Arenas, E. (2010). Emerging roles of Wnts in the adult nervous system. Nat Rev Neurosci *11*, 77-86.

Innocenti, G.M., Ansermet, F., and Parnas, J. (2003). Schizophrenia, neurodevelopment and corpus callosum. Mol Psychiatry *8*, 261-274.

Inoue, A., and Sanes, J.R. (1997). Lamina-specific connectivity in the brain: regulation by N-cadherin, neurotrophins, and glycoconjugates. Science *276*, 1428-1431.

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T.W., and Sudhof, T.C. (1997). Binding of neuroligins to PSD-95. Science *277*, 1511-1515.

Irwin, S.A., Galvez, R., and Greenough, W.T. (2000). Dendritic spine structural anomalies in fragile-X mental retardation syndrome. Cereb Cortex *10*, 1038-1044.

Isaac, J.T., Crair, M.C., Nicoll, R.A., and Malenka, R.C. (1997). Silent synapses during development of thalamocortical inputs. Neuron *18*, 269-280.

Ishikawa, T., Gotoh, N., Murayama, C., Abe, T., Iwashita, M., Matsuzaki, F., Suzuki, T., and Yamamoto, T. (2011). IgSF molecule MDGA1 is involved in radial migration and positioning of a subset of cortical upper-layer neurons. Dev Dyn *240*, 96-107.

Jacobsen, L.K., Picciotto, M.R., Heath, C.J., Mencl, W.E., and Gelernter, J. (2009). Allelic variation of calsyntenin 2 (CLSTN2) modulates the impact of developmental tobacco smoke exposure on mnemonic processing in adolescents. Biol Psychiatry *65*, 671-679.

Jacobson, C., Cote, P.D., Rossi, S.G., Rotundo, R.L., and Carbonetto, S. (2001). The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. J Cell Biol *152*, 435-450.

Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I.C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C.*, et al.* (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nature genetics *34*, 27-29.

Jamain, S., Radyushkin, K., Hammerschmidt, K., Granon, S., Boretius, S., Varoqueaux, F., Ramanantsoa, N., Gallego, J., Ronnenberg, A., Winter, D.*, et al.* (2008). Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. Proc Natl Acad Sci U S A *105*, 1710-1715.

Jedlicka, P., Hoon, M., Papadopoulos, T., Vlachos, A., Winkels, R., Poulopoulos, A., Betz, H., Deller, T., Brose, N., Varoqueaux, F.*, et al.* (2011). Increased dentate gyrus excitability in neuroligin-2-deficient mice in vivo. Cereb Cortex *21*, 357-367.

Jiang, Y.H., Armstrong, D., Albrecht, U., Atkins, C.M., Noebels, J.L., Eichele, G., Sweatt, J.D., and Beaudet, A.L. (1998). Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. Neuron *21*, 799-811.

Jiang, Y.H., and Beaudet, A.L. (2004). Human disorders of ubiquitination and proteasomal degradation. Curr Opin Pediatr *16*, 419-426.

Joo, J.Y., Lee, S.J., Uemura, T., Yoshida, T., Yasumura, M., Watanabe, M., and Mishina, M. (2011). Differential interactions of cerebellin precursor protein (Cbln) subtypes and neurexin variants for synapse formation of cortical neurons. Biochem Biophys Res Commun *406*, 627-632.

Jou, T.S., Stewart, D.B., Stappert, J., Nelson, W.J., and Marrs, J.A. (1995). Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. Proc Natl Acad Sci U S A *92*, 5067-5071.

Jugloff, D.G., Vandamme, K., Logan, R., Visanji, N.P., Brotchie, J.M., and Eubanks, J.H. (2008). Targeted delivery of an Mecp2 transgene to forebrain neurons improves the behavior of female Mecp2-deficient mice. Hum Mol Genet *17*, 1386-1396.

Juttner, R., More, M.I., Das, D., Babich, A., Meier, J., Henning, M., Erdmann, B., Mu Ller, E.C., Otto, A., Grantyn, R.*, et al.* (2005). Impaired synapse function during postnatal development in the absence of CALEB, an EGF-like protein processed by neuronal activity. Neuron *46*, 233-245.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nature Protocols *1*, 2406-2415.

Kahler, A.K., Djurovic, S., Kulle, B., Jonsson, E.G., Agartz, I., Hall, H., Opjordsmoen, S., Jakobsen, K.D., Hansen, T., Melle, I.*, et al.* (2008). Association analysis of schizophrenia on 18 genes involved in neuronal migration: MDGA1 as a new susceptibility gene. Am J Med Genet B Neuropsychiatr Genet *147B*, 1089-1100.

Kang, Y., Zhang, X., Dobie, F., Wu, H., and Craig, A.M. (2008). Induction of GABAergic postsynaptic differentiation by alpha-neurexins. J Biol Chem *283*, 2323-2334.

Kapur, S., Agid, O., Mizrahi, R., and Li, M. (2006). How antipsychotics work-from receptors to reality. NeuroRx *3*, 10-21.

Karagogeos, D. (2003). Neural GPI-anchored cell adhesion molecules. Front Biosci *8*, s1304-1320.

Kashiwabuchi, N., Ikeda, K., Araki, K., Hirano, T., Shibuki, K., Takayama, C., Inoue, Y., Kutsuwada, T., Yagi, T., Kang, Y.*, et al.* (1995). Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice. Cell *81*, 245-252.

Kattenstroth, G., Tantalaki, E., Sudhof, T.C., Gottmann, K., and Missler, M. (2004). Postsynaptic N-methyl-D-aspartate receptor function requires alpha-neurexins. Proc Natl Acad Sci U S A *101*, 2607-2612.

Katz, B., and Miledi, R. (1965). Release of acetylcholine from a nerve terminal by electric pulses of variable strength and duration. Nature *207*, 1097-1098.

Katz, L.C., and Shatz, C.J. (1996). Synaptic activity and the construction of cortical circuits. Science *274*, 1133-1138.

Kayser, M.S., McClelland, A.C., Hughes, E.G., and Dalva, M.B. (2006). Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors. J Neurosci *26*, 12152-12164.

Kayser, M.S., Nolt, M.J., and Dalva, M.B. (2008). EphB receptors couple dendritic filopodia motility to synapse formation. Neuron *59*, 56-69.

Kennedy, T.E. (2000). Cellular mechanisms of netrin function: long-range and short-range actions. Biochem Cell Biol *78*, 569-575.

Kenwrick, S., Watkins, A., and De Angelis, E. (2000). Neural cell recognition molecule L1: relating biological complexity to human disease mutations. Hum Mol Genet *9*, 879-886.

Keshavan, M.S., Anderson, S., and Pettegrew, J.W. (1994). Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? The Feinberg hypothesis revisited. J Psychiatr Res *28*, 239-265.

Kim, J., Jung, S.Y., Lee, Y.K., Park, S., Choi, J.S., Lee, C.J., Kim, H.S., Choi, Y.B., Scheiffele, P., Bailey, C.H.*, et al.* (2008). Neuroligin-1 is required for normal expression of LTP and associative fear memory in the amygdala of adult animals. Proc Natl Acad Sci U S A *105*, 9087-9092.

Kim, S., Burette, A., Chung, H.S., Kwon, S.K., Woo, J., Lee, H.W., Kim, K., Kim, H., Weinberg, R.J., and Kim, E. (2006). NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. Nat Neurosci *9*, 1294-1301.

King, B.H., and Lord, C. (2011). Is schizophrenia on the autism spectrum? Brain Res *1380*, 34-41.

Kirov, G., Gumus, D., Chen, W., Norton, N., Georgieva, L., Sari, M., O'Donovan, M.C., Erdogan, F., Owen, M.J., Ropers, H.H.*, et al.* (2008). Comparative genome hybridization suggests a role for NRXN1 and APBA2 in schizophrenia. Hum Mol Genet *17*, 458-465.

Klassen, M.P., and Shen, K. (2007). Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in C. elegans. Cell *130*, 704-716.

Ko, J., Fuccillo, M.V., Malenka, R.C., and Sudhof, T.C. (2009). LRRTM2 functions as a neurexin ligand in promoting excitatory synapse formation. Neuron *64*, 791-798.

Ko, J., Kim, S., Chung, H.S., Kim, K., Han, K., Kim, H., Jun, H., Kaang, B.K., and Kim, E. (2006). SALM synaptic cell adhesion-like molecules regulate the differentiation of excitatory synapses. Neuron *50*, 233-245.

Ko, J., Soler-Llavina, G.J., Fuccillo, M.V., Malenka, R.C., and Sudhof, T.C. (2011). Neuroligins/LRRTMs prevent activity- and Ca2+/calmodulin-dependent synapse elimination in cultured neurons. J Cell Biol *194*, 323-334.

Koch, S.M., and Ullian, E.M. (2010). Neuronal pentraxins mediate silent synapse conversion in the developing visual system. J Neurosci *30*, 5404-5414.

Koehnke, J., Jin, X., Budreck, E.C., Posy, S., Scheiffele, P., Honig, B., and Shapiro, L. (2008). Crystal structure of the extracellular cholinesterase-like domain from neuroligin-2. Proc Natl Acad Sci U S A *105*, 1873-1878.

Koehnke, J., Katsamba, P.S., Ahlsen, G., Bahna, F., Vendome, J., Honig, B., Shapiro, L., and Jin, X. (2010). Splice form dependence of beta-neurexin/neuroligin binding interactions. Neuron *67*, 61-74.

Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., and Yagi, T. (1998). Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. Neuron *20*, 1137-1151.

Konecna, A., Frischknecht, R., Kinter, J., Ludwig, A., Steuble, M., Meskenaite, V., Indermuhle, M., Engel, M., Cen, C., Mateos, J.M.*, et al.* (2006). Calsyntenin-1 docks vesicular cargo to kinesin-1. Mol Biol Cell *17*, 3651-3663.

Konno, T., Hata, S., Hamada, Y., Horikoshi-Sakuraba, Y., Nakaya, T., Saito, Y., Yamamoto, T., Maeda, M., Ikeuchi, T., Gandy, S.*, et al.* (2011). Coordinated increase of gamma-secretase reaction products in the plasma of some female Japanese sporadic Alzheimer's disease patients: quantitative analysis of p3-Alcalpha with a new ELISA system. Mol Neurodegener *6*, 76.

Kootz, J.P., Marinelli, B., and Cohen, D.J. (1981). Sensory receptor sensitivity in autistic children: response times to proximal and distal stimulation. Arch Gen Psychiatry *38*, 271-273.

Kullander, K., and Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol *3*, 475-486.

Kwon, S.K., Woo, J., Kim, S.Y., Kim, H., and Kim, E. (2010). Trans-synaptic adhesions between netrin-G ligand-3 (NGL-3) and receptor tyrosine phosphatases LAR, protein-tyrosine phosphatase delta (PTPdelta), and PTPsigma via specific domains regulate excitatory synapse formation. J Biol Chem *285*, 13966-13978.

Lai, K.O., and Ip, N.Y. (2009). Synapse development and plasticity: roles of ephrin/Eph receptor signaling. Curr Opin Neurobiol *19*, 275-283.

Landis, D.M. (1988). Membrane and cytoplasmic structure at synaptic junctions in the mammalian central nervous system. J Electron Microsc Tech *10*, 129-151.

Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M.P., Raynaud, M., Ronce, N., Lemonnier, E., Calvas, P.*, et al.* (2004). X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. Am J Hum Genet *74*, 552-557.

Lauren, J., Airaksinen, M.S., Saarma, M., and Timmusk, T. (2003). A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system. Genomics *81*, 411-421.

Lawson-Yuen, A., Saldivar, J.S., Sommer, S., and Picker, J. (2008). Familial deletion within NLGN4 associated with autism and Tourette syndrome. Eur J Hum Genet *16*, 614-618.

LeBlanc, J.J., and Fagiolini, M. (2011). Autism: a "critical period" disorder? Neural Plast *2011*, 921680.

Lee, C.H., Herman, T., Clandinin, T.R., Lee, R., and Zipursky, S.L. (2001). N-cadherin regulates target specificity in the Drosophila visual system. Neuron *30*, 437-450.

Lee, R.C., Clandinin, T.R., Lee, C.H., Chen, P.L., Meinertzhagen, I.A., and Zipursky, S.L. (2003). The protocadherin Flamingo is required for axon target selection in the Drosophila visual system. Nat Neurosci *6*, 557-563.

Lee, S.H., Peng, I.F., Ng, Y.G., Yanagisawa, M., Bamji, S.X., Elia, L.P., Balsamo, J., Lilien, J., Anastasiadis, P.Z., Ullian, E.M.*, et al.* (2008a). Synapses are regulated by the cytoplasmic tyrosine kinase Fer in a pathway mediated by p120catenin, Fer, SHP-2, and beta-catenin. J Cell Biol *183*, 893-908.

Lee, T.W., Tsang, V.W., and Birch, N.P. (2008b). Synaptic plasticity-associated proteases and protease inhibitors in the brain linked to the processing of extracellular matrix and cell adhesion molecules. Neuron Glia Biol *4*, 223-234.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J.*, et al.* (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168-176.

Leone, P., Comoletti, D., Ferracci, G., Conrod, S., Garcia, S.U., Taylor, P., Bourne, Y., and Marchot, P. (2010). Structural insights into the exquisite selectivity of neurexin/neuroligin synaptic interactions. Embo J *29*, 2461-2471.

Leonoudakis, D., Conti, L.R., Radeke, C.M., McGuire, L.M., and Vandenberg, C.A. (2004). A multiprotein trafficking complex composed of SAP97, CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. J Biol Chem *279*, 19051-19063.

Levi, S., Grady, R.M., Henry, M.D., Campbell, K.P., Sanes, J.R., and Craig, A.M. (2002). Dystroglycan is selectively associated with inhibitory GABAergic synapses but is dispensable for their differentiation. J Neurosci *22*, 4274-4285.

Levinson, J.N., Chery, N., Huang, K., Wong, T.P., Gerrow, K., Kang, R., Prange, O., Wang, Y.T., and El-Husseini, A. (2005). Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. J Biol Chem *280*, 17312-17319.

Levinson, J.N., and El-Husseini, A. (2005). Building excitatory and inhibitory synapses: balancing neuroligin partnerships. Neuron *48*, 171-174.

Levinson, J.N., and El-Husseini, A. (2007). A crystal-clear interaction: relating neuroligin/neurexin complex structure to function at the synapse. Neuron *56*, 937-939.

Levy, S.E., Mandell, D.S., and Schultz, R.T. (2009). Autism. Lancet *374*, 1627-1638.

Li, A.J., Suzuki, S., Suzuki, M., Mizukoshi, E., and Imamura, T. (2002). Fibroblast growth factor-2 increases functional excitatory synapses on hippocampal neurons. Eur J Neurosci *16*, 1313-1324.

Li, J., Liu, J., Feng, G., Li, T., Zhao, Q., Li, Y., Hu, Z., Zheng, L., Zeng, Z., He, L.*, et al.* (2011). The MDGA1 gene confers risk to schizophrenia and bipolar disorder. Schizophr Res *125*, 194-200.

Linhoff, M.W. (2008). Expression Screening for Proteins Involved in CNS Presynaptic Differentiation and Initial Characterization of the Synaptogenic LRRTM Protein Family (St. Louis, Washington University in St. Louis).

Linhoff, M.W., Lauren, J., Cassidy, R.M., Dobie, F.A., Takahashi, H., Nygaard, H.B., Airaksinen, M.S., Strittmatter, S.M., and Craig, A.M. (2009). An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. Neuron *61*, 734-749.

Litwack, E.D., Babey, R., Buser, R., Gesemann, M., and O'Leary, D.D. (2004). Identification and characterization of two novel brain-derived immunoglobulin superfamily members with a unique structural organization. Mol Cell Neurosci *25*, 263-274.

Ludwig, A., Blume, J., Diep, T.M., Yuan, J., Mateos, J.M., Leuthauser, K., Steuble, M., Streit, P., and Sonderegger, P. (2009). Calsyntenins mediate TGN exit of APP in a kinesin-1-dependent manner. Traffic *10*, 572-589.

Mabb, A.M., and Ehlers, M.D. (2010). Ubiquitination in postsynaptic function and plasticity. Annu Rev Cell Dev Biol *26*, 179-210.

Madani, R., Hulo, S., Toni, N., Madani, H., Steimer, T., Muller, D., and Vassalli, J.D. (1999). Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. Embo J *18*, 3007-3012.

Mah, W., Ko, J., Nam, J., Han, K., Chung, W.S., and Kim, E. (2010). Selected SALM (synaptic adhesion-like molecule) family proteins regulate synapse formation. J Neurosci *30*, 5559-5568.

Malinow, R., and Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci *25*, 103-126.

Maretzky, T., Schulte, M., Ludwig, A., Rose-John, S., Blobel, C., Hartmann, D., Altevogt, P., Saftig, P., and Reiss, K. (2005). L1 is sequentially processed by two differently activated metalloproteases and presenilin/gamma-secretase and regulates neural cell adhesion, cell migration, and neurite outgrowth. Mol Cell Biol *25*, 9040-9053.

Marin, O., and Rubenstein, J.L. (2003). Cell migration in the forebrain. Annu Rev Neurosci *26*, 441-483.

Marshall, C.R., Noor, A., Vincent, J.B., Lionel, A.C., Feuk, L., Skaug, J., Shago, M., Moessner, R., Pinto, D., Ren, Y.*, et al.* (2008). Structural variation of chromosomes in autism spectrum disorder. Am J Hum Genet *82*, 477-488.

Matsuda, K., Miura, E., Miyazaki, T., Kakegawa, W., Emi, K., Narumi, S., Fukazawa, Y., Ito-Ishida, A., Kondo, T., Shigemoto, R.*, et al.* (2010). Cbln1 is a ligand for an orphan glutamate receptor delta2, a bidirectional synapse organizer. Science *328*, 363-368.

Matsuda, K., and Yuzaki, M. (2011). Cbln family proteins promote synapse formation by regulating distinct neurexin signaling pathways in various brain regions. Eur J Neurosci *33*, 1447-1461.

Matteoli, M., Takei, K., Perin, M.S., Sudhof, T.C., and De Camilli, P. (1992). Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. J Cell Biol *117*, 849-861.

Mauch, D.H., Nagler, K., Schumacher, S., Goritz, C., Muller, E.C., Otto, A., and Pfrieger, F.W. (2001). CNS synaptogenesis promoted by glia-derived cholesterol. Science *294*, 1354-1357.

Maximov, A., Sudhof, T.C., and Bezprozvanny, I. (1999). Association of neuronal calcium channels with modular adaptor proteins. J Biol Chem *274*, 24453-24456.

McAllister, A.K. (2007). Dynamic aspects of CNS synapse formation. Annu Rev Neurosci *30*, 425-450.

McClelland, A.C., Hruska, M., Coenen, A.J., Henkemeyer, M., and Dalva, M.B. (2010). Trans-synaptic EphB2-ephrin-B3 interaction regulates excitatory synapse density by inhibition of postsynaptic MAPK signaling. Proc Natl Acad Sci U S A *107*, 8830-8835.

Meyer, G., Varoqueaux, F., Neeb, A., Oschlies, M., and Brose, N. (2004). The complexity of PDZ domain-mediated interactions at glutamatergic synapses: a case study on neuroligin. Neuropharmacology *47*, 724-733.

Mi, R., Tang, X., Sutter, R., Xu, D., Worley, P., and O'Brien, R.J. (2002). Differing mechanisms for glutamate receptor aggregation on dendritic spines and shafts in cultured hippocampal neurons. J Neurosci *22*, 7606-7616.

Micheva, K.D., and Beaulieu, C. (1996). Quantitative aspects of synaptogenesis in the rat barrel field cortex with special reference to GABA circuitry. J Comp Neurol *373*, 340-354.

Miller, M.T., Mileni, M., Comoletti, D., Stevens, R.C., Harel, M., and Taylor, P. (2011). The crystal structure of the alpha-neurexin-1 extracellular region reveals a hinge point for mediating synaptic adhesion and function. Structure *19*, 767-778.

Ming, G.L., and Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci *28*, 223-250.

Missler, M., Hammer, R.E., and Sudhof, T.C. (1998). Neurexophilin binding to alpha-neurexins. A single LNS domain functions as an independently folding ligand-binding unit. J Biol Chem *273*, 34716-34723.

Missler, M., and Sudhof, T.C. (1998). Neurexins: three genes and 1001 products. Trends Genet *14*, 20-26.

Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R.E., Gottmann, K., and Sudhof, T.C. (2003). Alpha-neurexins couple Ca2+ channels to synaptic vesicle exocytosis. Nature *423*, 939-948.

Mitchell, K.J. (2011). The genetics of neurodevelopmental disease. Curr Opin Neurobiol *21*, 197-203.

Moessner, R., Marshall, C.R., Sutcliffe, J.S., Skaug, J., Pinto, D., Vincent, J., Zwaigenbaum, L., Fernandez, B., Roberts, W., Szatmari, P.*, et al.* (2007). Contribution of SHANK3 mutations to autism spectrum disorder. Am J Hum Genet *81*, 1289-1297.

Moore, S.A., Saito, F., Chen, J., Michele, D.E., Henry, M.D., Messing, A., Cohn, R.D., Ross-Barta, S.E., Westra, S., Williamson, R.A.*, et al.* (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. Nature *418*, 422-425.

Moretti, P., Bouwknecht, J.A., Teague, R., Paylor, R., and Zoghbi, H.Y. (2005). Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. Hum Mol Genet *14*, 205-220.

Moretti, P., Levenson, J.M., Battaglia, F., Atkinson, R., Teague, R., Antalffy, B., Armstrong, D., Arancio, O., Sweatt, J.D., and Zoghbi, H.Y. (2006). Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. J Neurosci *26*, 319-327.

Morrow, E.M., Yoo, S.Y., Flavell, S.W., Kim, T.K., Lin, Y., Hill, R.S., Mukaddes, N.M., Balkhy, S., Gascon, G., Hashmi, A.*, et al.* (2008). Identifying autism loci and genes by tracing recent shared ancestry. Science *321*, 218-223.

Mueser, K.T., and McGurk, S.R. (2004). Schizophrenia. The Lancet *363*, 2063-2072.

Mukherjee, K., Sharma, M., Urlaub, H., Bourenkov, G.P., Jahn, R., Sudhof, T.C., and Wahl, M.C. (2008). CASK Functions as a Mg2+-independent neurexin kinase. Cell *133*, 328-339.

Murase, S., Mosser, E., and Schuman, E.M. (2002). Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. Neuron *35*, 91-105.

Najm, J., Horn, D., Wimplinger, I., Golden, J.A., Chizhikov, V.V., Sudi, J., Christian, S.L., Ullmann, R., Kuechler, A., Haas, C.A.*, et al.* (2008). Mutations of CASK cause an X-linked brain malformation phenotype with microcephaly and hypoplasia of the brainstem and cerebellum. Nature genetics *40*, 1065-1067.

Nam, C.I., and Chen, L. (2005). Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. Proc Natl Acad Sci U S A *102*, 6137-6142.

Niederkofler, V., Baeriswyl, T., Ott, R., and Stoeckli, E.T. (2010). Nectin-like molecules/SynCAMs are required for post-crossing commissural axon guidance. Development *137*, 427-435.

Nishimura, W., Yao, I., Iida, J., Tanaka, N., and Hata, Y. (2002). Interaction of synaptic scaffolding molecule and Beta -catenin. J Neurosci *22*, 757-765.

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene *108*, 193-199.

Nuriya, M., and Huganir, R.L. (2006). Regulation of AMPA receptor trafficking by N-cadherin. J Neurochem *97*, 652-661.

O'Brien, R.J., Xu, D., Petralia, R.S., Steward, O., Huganir, R.L., and Worley, P. (1999). Synaptic clustering of AMPA receptors by the extracellular immediate-early gene product Narp. Neuron *23*, 309-323.

O'Donnell, M., Chance, R.K., and Bashaw, G.J. (2009). Axon growth and guidance: receptor regulation and signal transduction. Annu Rev Neurosci *32*, 383-412.

Oertel, W.H., and Mugnaini, E. (1984). Immunocytochemical studies of GABAergic neurons in rat basal ganglia and their relations to other neuronal systems. Neurosci Lett *47*, 233-238.

Palay, S.L. (1956a). Structure and function in the neuron. Prog Neurobiol *1*, 64-82.

Palay, S.L. (1956b). Synapses in the central nervous system. J Biophys Biochem Cytol *2*, 193-202.

Pan, F., and Gan, W.B. (2008). Two-photon imaging of dendritic spine development in the mouse cortex. Dev Neurobiol *68*, 771-778.

Paoletti, P., and Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol *7*, 39-47.

Papassotiropoulos, A., Stephan, D.A., Huentelman, M.J., Hoerndli, F.J., Craig, D.W., Pearson, J.V., Huynh, K.D., Brunner, F., Corneveaux, J., Osborne, D.*, et al.* (2006). Common Kibra alleles are associated with human memory performance. Science *314*, 475-478.

Park, J., Liu, B., Chen, T., Li, H., Hu, X., Gao, J., Zhu, Y., Zhu, Q., Qiang, B., Yuan, J.*, et al.* (2008). Disruption of Nectin-like 1 cell adhesion molecule leads to delayed axonal myelination in the CNS. J Neurosci *28*, 12815-12819.

Pasterkamp, R.J., and Kolodkin, A.L. (2003). Semaphorin junction: making tracks toward neural connectivity. Curr Opin Neurobiol *13*, 79-89.

Peca, J., Feliciano, C., Ting, J.T., Wang, W., Wells, M.F., Venkatraman, T.N., Lascola, C.D., Fu, Z., and Feng, G. (2011a). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature *472*, 437-442.

Peca, J., Ting, J., and Feng, G. (2011b). SnapShot: Autism and the synapse. Cell *147*, 706, 706 e701.

Perego, C., Vanoni, C., Massari, S., Longhi, R., and Pietrini, G. (2000). Mammalian LIN-7 PDZ proteins associate with beta-catenin at the cell-cell junctions of epithelia and neurons. Embo J *19*, 3978-3989.

Peters, A., and Palay, S.L. (1996). The morphology of synapses. J Neurocytol *25*, 687-700.

Peters, A., Palay, S.L., and Webster, H.d. (1991). The fine structure of the nervous system : neurons and their supporting cells, 3rd edn (New York, Oxford University Press).

Petrenko, A.G., Ullrich, B., Missler, M., Krasnoperov, V., Rosahl, T.W., and Sudhof, T.C. (1996). Structure and evolution of neurexophilin. J Neurosci *16*, 4360-4369.

Phillips, G.R., Huang, J.K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, W.S., Arndt, K., Frank, M., Gordon, R.E.*, et al.* (2001). The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. Neuron *32*, 63-77.

Phillips, G.R., Tanaka, H., Frank, M., Elste, A., Fidler, L., Benson, D.L., and Colman, D.R. (2003). Gamma-protocadherins are targeted to subsets of synapses and intracellular organelles in neurons. J Neurosci *23*, 5096-5104.

Pickard, B. (2011). Progress in defining the biological causes of schizophrenia. Expert Rev Mol Med *13*, e25.

Pierce, J.P., and Mendell, L.M. (1993). Quantitative ultrastructure of Ia boutons in the ventral horn: scaling and positional relationships. J Neurosci *13*, 4748-4763.

Pillai, A.M., Garcia-Fresco, G.P., Sousa, A.D., Dupree, J.L., Philpot, B.D., and Bhat, M.A. (2007). No effect of genetic deletion of contactin-associated protein (CASPR) on axonal orientation and synaptic plasticity. J Neurosci Res *85*, 2318-2331.

Pinto, D., Pagnamenta, A.T., Klei, L., Anney, R., Merico, D., Regan, R., Conroy, J., Magalhaes, T.R., Correia, C., Abrahams, B.S.*, et al.* (2010). Functional impact of global rare copy number variation in autism spectrum disorders. Nature *466*, 368-372.

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K.*, et al.* (2009). Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. Neuron *63*, 628-642.

Prange, O., Wong, T.P., Gerrow, K., Wang, Y.T., and El-Husseini, A. (2004). A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc Natl Acad Sci U S A *101*, 13915-13920.

Preuschhof, C., Heekeren, H.R., Li, S.C., Sander, T., Lindenberger, U., and Backman, L. (2010). KIBRA and CLSTN2 polymorphisms exert interactive effects on human episodic memory. Neuropsychologia *48*, 402-408.

Price, D.L., Sisodia, S.S., and Borchelt, D.R. (1998). Genetic neurodegenerative diseases: the human illness and transgenic models. Science *282*, 1079-1083.

Pulido, R., Serra-Pages, C., Tang, M., and Streuli, M. (1995). The LAR/PTP delta/PTP sigma subfamily of transmembrane protein-tyrosine-phosphatases: multiple human LAR, PTP delta, and PTP sigma isoforms are expressed in a tissue-specific manner and associate with the LAR-interacting protein LIP.1. Proc Natl Acad Sci U S A *92*, 11686-11690.

Qian, Z., Gilbert, M.E., Colicos, M.A., Kandel, E.R., and Kuhl, D. (1993). Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature *361*, 453-457.

Reiss, K., Maretzky, T., Ludwig, A., Tousseyn, T., de Strooper, B., Hartmann, D., and Saftig, P. (2005). ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. Embo J *24*, 742-752.

Reiss, K., and Saftig, P. (2009). The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. Semin Cell Dev Biol *20*, 126-137.

Ringman, J.M., Schulman, H., Becker, C., Jones, T., Bai, Y., Immermann, F., Cole, G., Sokolow, S., Gylys, K., Geschwind, D.H.*, et al.* (2012). Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer disease mutations. Arch Neurol *69*, 96-104.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature *397*, 251-255.

Robbins, E.M., Krupp, A.J., Perez de Arce, K., Ghosh, A.K., Fogel, A.I., Boucard, A., Sudhof, T.C., Stein, V., and Biederer, T. (2010). SynCAM 1 adhesion dynamically regulates synapse number and impacts plasticity and learning. Neuron *68*, 894-906.

Robertson, H.R., and Feng, G. (2011). Annual Research Review: Transgenic mouse models of childhood-onset psychiatric disorders. J Child Psychol Psychiatry *52*, 442-475.

Roohi, J., Montagna, C., Tegay, D.H., Palmer, L.E., DeVincent, C., Pomeroy, J.C., Christian, S.L., Nowak, N., and Hatchwell, E. (2009). Disruption of contactin 4 in three subjects with autism spectrum disorder. J Med Genet *46*, 176-182.

Rowen, L., Young, J., Birditt, B., Kaur, A., Madan, A., Philipps, D.L., Qin, S., Minx, P., Wilson, R.K., Hood, L.*, et al.* (2002). Analysis of the human neurexin genes: alternative splicing and the generation of protein diversity. Genomics *79*, 587-597.

Rudenko, G., Hohenester, E., and Muller, Y.A. (2001). LG/LNS domains: multiple functions -- one business end? Trends Biochem Sci *26*, 363-368.

Sahores, M., Gibb, A., and Salinas, P.C. (2010). Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. Development *137*, 2215-2225.

Salinas, P.C., and Zou, Y. (2008). Wnt signaling in neural circuit assembly. Annu Rev Neurosci *31*, 339-358.

Sara, Y., Biederer, T., Atasoy, D., Chubykin, A., Mozhayeva, M.G., Sudhof, T.C., and Kavalali, E.T. (2005). Selective capability of SynCAM and neuroligin for functional synapse assembly. J Neurosci *25*, 260-270.

Scheiffele, P. (2003). Cell-cell signaling during synapse formation in the CNS. Annu Rev Neurosci *26*, 485-508.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell *101*, 657-669.

Schikorski, T., and Stevens, C.F. (1997). Quantitative ultrastructural analysis of hippocampal excitatory synapses. J Neurosci *17*, 5858-5867.

Schmitz, C., and Rezaie, P. (2008). The neuropathology of autism: where do we stand? Neuropathol Appl Neurobiol *34*, 4-11.

Seabold, G.K., Wang, P.Y., Chang, K., Wang, C.Y., Wang, Y.X., Petralia, R.S., and Wenthold, R.J. (2008). The SALM family of adhesion-like molecules forms heteromeric and homomeric complexes. J Biol Chem *283*, 8395-8405.

Seals, D.F., and Courtneidge, S.A. (2003). The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes & Development *17*, 7-30.

Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. Physiol Rev *81*, 741-766.

Shahbazian, M., Young, J., Yuva-Paylor, L., Spencer, C., Antalffy, B., Noebels, J., Armstrong, D., Paylor, R., and Zoghbi, H. (2002). Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron *35*, 243-254.

Shapira, M., Zhai, R.G., Dresbach, T., Bresler, T., Torres, V.I., Gundelfinger, E.D., Ziv, N.E., and Garner, C.C. (2003). Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. Neuron *38*, 237-252.

Shapiro, L., and Colman, D.R. (1999). The diversity of cadherins and implications for a synaptic adhesive code in the CNS. Neuron *23*, 427-430.

Shen, K.C., Kuczynska, D.A., Wu, I.J., Murray, B.H., Sheckler, L.R., and Rudenko, G. (2008). Regulation of neurexin 1beta tertiary structure and ligand binding through alternative splicing. Structure *16*, 422-431.

Sheng, M. (2001). Molecular organization of the postsynaptic specialization. Proc Natl Acad Sci U S A *98*, 7058-7061.

Sheng, M., and Hoogenraad, C.C. (2007). The postsynaptic architecture of excitatory synapses: a more quantitative view. Annu Rev Biochem *76*, 823-847.

Sheng, M., and Kim, E. (2000). The Shank family of scaffold proteins. J Cell Sci *113 (Pt 11)*, 1851-1856.

Shipman, S.L., Schnell, E., Hirai, T., Chen, B.S., Roche, K.W., and Nicoll, R.A. (2011). Functional dependence of neuroligin on a new non-PDZ intracellular domain. Nat Neurosci *14*, 718-726.

Sia, G.M., Beique, J.C., Rumbaugh, G., Cho, R., Worley, P.F., and Huganir, R.L. (2007). Interaction of the N-terminal domain of the AMPA receptor GluR4 subunit with the neuronal pentraxin NP1 mediates GluR4 synaptic recruitment. Neuron *55*, 87-102.

Siddiqui, T.J., and Craig, A.M. (2011). Synaptic organizing complexes. Curr Opin Neurobiol *21*, 132-143.

Siddiqui, T.J., Pancaroglu, R., Kang, Y., Rooyakkers, A., and Craig, A.M. (2010). LRRTMs and neuroligins bind neurexins with a differential code to cooperate in glutamate synapse development. J Neurosci *30*, 7495-7506.

Sigrist, S.J., and Schmitz, D. (2011). Structural and functional plasticity of the cytoplasmic active zone. Curr Opin Neurobiol *21*, 144-150.

Skutella, T., and Nitsch, R. (2001). New molecules for hippocampal development. Trends Neurosci *24*, 107-113.

Soler-Llavina, G.J., Fuccillo, M.V., Ko, J., Sudhof, T.C., and Malenka, R.C. (2011). The neurexin ligands, neuroligins and leucine-rich repeat transmembrane proteins, perform convergent and divergent synaptic functions in vivo. Proc Natl Acad Sci U S A *108*, 16502-16509.

Somogyi, P., Tamas, G., Lujan, R., and Buhl, E.H. (1998). Salient features of synaptic organisation in the cerebral cortex. Brain Res Brain Res Rev *26*, 113-135.

Song, J.Y., Ichtchenko, K., Sudhof, T.C., and Brose, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc Natl Acad Sci U S A *96*, 1100-1105.

Sousa, I., Clark, T.G., Holt, R., Pagnamenta, A.T., Mulder, E.J., Minderaa, R.B., Bailey, A.J., Battaglia, A., Klauck, S.M., Poustka, F.*, et al.* (2010). Polymorphisms in leucine-rich repeat genes are associated with autism spectrum disorder susceptibility in populations of European ancestry. Mol Autism *1*, 7.

Squire, L.R., and Zola, S.M. (1996). Structure and function of declarative and nondeclarative memory systems. Proc Natl Acad Sci U S A *93*, 13515-13522.

Stagi, M., Fogel, A.I., and Biederer, T. (2010). SynCAM 1 participates in axo-dendritic contact assembly and shapes neuronal growth cones. Proc Natl Acad Sci U S A *107*, 7568-7573.

Stan, A., Pielarski, K.N., Brigadski, T., Wittenmayer, N., Fedorchenko, O., Gohla, A., Lessmann, V., Dresbach, T., and Gottmann, K. (2010). Essential cooperation of N-cadherin and neuroligin-1 in the transsynaptic control of vesicle accumulation. Proc Natl Acad Sci U S A *107*, 11116-11121.

State, M.W., and Levitt, P. (2011). The conundrums of understanding genetic risks for autism spectrum disorders. Nat Neurosci *14*, 1499-1506.

Steuble, M., Gerrits, B., Ludwig, A., Mateos, J.M., Diep, T.M., Tagaya, M., Stephan, A., Schatzle, P., Kunz, B., Streit, P.*, et al.* (2010). Molecular characterization of a trafficking organelle: dissecting the axonal paths of calsyntenin-1 transport vesicles. Proteomics *10*, 3775-3788.

Stryker, E., and Johnson, K.G. (2007). LAR, liprin alpha and the regulation of active zone morphogenesis. J Cell Sci *120*, 3723-3728.

Sudhof, T.C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. Nature *455*, 903-911.

Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Sudhof, T.C. (2001). A stoichiometric complex of neurexins and dystroglycan in brain. J Cell Biol *154*, 435-445.

Sun, Y., Aiga, M., Yoshida, E., Humbert, P.O., and Bamji, S.X. (2009). Scribble interacts with beta-catenin to localize synaptic vesicles to synapses. Mol Biol Cell *20*, 3390-3400.

Szatmari, P., Paterson, A.D., Zwaigenbaum, L., Roberts, W., Brian, J., Liu, X.Q., Vincent, J.B., Skaug, J.L., Thompson, A.P., Senman, L.*, et al.* (2007). Mapping autism risk loci using genetic linkage and chromosomal rearrangements. Nature genetics *39*, 319-328.

Tabuchi, K., Blundell, J., Etherton, M.R., Hammer, R.E., Liu, X., Powell, C.M., and Sudhof, T.C. (2007). A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science *318*, 71-76.

Tabuchi, K., and Sudhof, T.C. (2002). Structure and evolution of neurexin genes: insight into the mechanism of alternative splicing. Genomics *79*, 849-859.

Takahashi, H., Arstikaitis, P., Prasad, T., Bartlett, T.E., Wang, Y.T., Murphy, T.H., and Craig, A.M. (2011). Postsynaptic TrkC and Presynaptic PTPσ Function as a Bidirectional Excitatory Synaptic Organizing Complex. Neuron *69*, 287-303.

Takahashi, H., Katayama, K.I., Sohya, K., Miyamoto, H., Prasad, T., Matsumoto, Y., Ota, M., Yasuda, H., Tsumoto, T., Aruga, J.*, et al.* (2012). Selective control of inhibitory synapse development by Slitrk3-PTPdelta trans-synaptic interaction. Nat Neurosci.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science *251*, 1451-1455.

Takeuchi, A., Hamasaki, T., Litwack, E.D., and O'Leary, D.D. (2007a). Novel IgCAM, MDGA1, expressed in unique cortical area- and layer-specific patterns and transiently by distinct forebrain populations of Cajal-Retzius neurons. Cereb Cortex *17*, 1531-1541.

Takeuchi, A., Hamasaki, T., Litwack, E.D., and O'Leary, D.D. (2007b). Novel IgCAM, MDGA1, expressed in unique cortical area- and layer-specific patterns and transiently by distinct forebrain populations of Cajal-Retzius neurons. Cereb Cortex *17*, 1531-1541.

Takeuchi, A., and O'Leary, D.D. (2006). Radial migration of superficial layer cortical neurons controlled by novel Ig cell adhesion molecule MDGA1. J Neurosci *26*, 4460-4464.

Taniguchi, H., Gollan, L., Scholl, F.G., Mahadomrongkul, V., Dobler, E., Limthong, N., Peck, M., Aoki, C., and Scheiffele, P. (2007). Silencing of neuroligin function by postsynaptic neurexins. J Neurosci *27*, 2815-2824.

Terauchi, A., Johnson-Venkatesh, E.M., Toth, A.B., Javed, D., Sutton, M.A., and Umemori, H. (2010). Distinct FGFs promote differentiation of excitatory and inhibitory synapses. Nature *465*, 783-787.

Tessier-Lavigne, M. (2002). Wiring the brain: the logic and molecular mechanisms of axon guidance and regeneration. Harvey Lect *98*, 103-143.

Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., and Takeichi, M. (2002). Cadherin regulates dendritic spine morphogenesis. Neuron *35*, 77-89.

Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y., and Suzuki, T. (1999). Interaction of a neuron-specific protein containing PDZ domains with Alzheimer's amyloid precursor protein. J Biol Chem *274*, 2243-2254.

Tropea, D., Giacometti, E., Wilson, N.R., Beard, C., McCurry, C., Fu, D.D., Flannery, R., Jaenisch, R., and Sur, M. (2009). Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. Proc Natl Acad Sci U S A *106*, 2029-2034.

Turrigiano, G.G., and Nelson, S.B. (2004). Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci *5*, 97-107.

Uchida, N., Honjo, Y., Johnson, K.R., Wheelock, M.J., and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. J Cell Biol *135*, 767-779.

Uchida, Y., Nakano, S., Gomi, F., and Takahashi, H. (2011). Up-regulation of calsyntenin-3 by beta-amyloid increases vulnerability of cortical neurons. FEBS Lett *585*, 651-656.

Uemura, K., Kihara, T., Kuzuya, A., Okawa, K., Nishimoto, T., Ninomiya, H., Sugimoto, H., Kinoshita, A., and Shimohama, S. (2006). Characterization of sequential N-cadherin cleavage by ADAM10 and PS1. Neurosci Lett *402*, 278-283.

Uemura, T., Lee, S.J., Yasumura, M., Takeuchi, T., Yoshida, T., Ra, M., Taguchi, R., Sakimura, K., and Mishina, M. (2010). Trans-synaptic interaction of GluRdelta2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. Cell *141*, 1068-1079.

Ullian, E.M., Christopherson, K.S., and Barres, B.A. (2004). Role for glia in synaptogenesis. Glia *47*, 209-216.

Ullrich, B., Ushkaryov, Y.A., and Sudhof, T.C. (1995). Cartography of neurexins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. Neuron *14*, 497-507.

Umemori, H., Linhoff, M.W., Ornitz, D.M., and Sanes, J.R. (2004). FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. Cell *118*, 257-270.

Ushkaryov, Y.A., Petrenko, A.G., Geppert, M., and Sudhof, T.C. (1992). Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. Science *257*, 50-56.

Vaags, A.K., Lionel, A.C., Sato, D., Goodenberger, M., Stein, Q.P., Curran, S., Ogilvie, C., Ahn, J.W., Drmic, I., Senman, L.*, et al.* (2012). Rare deletions at the neurexin 3 locus in autism spectrum disorder. Am J Hum Genet *90*, 133-141.

Vagnoni, A., Rodriguez, L., Manser, C., De Vos, K.J., and Miller, C.C. (2011). Phosphorylation of kinesin light chain 1 at serine 460 modulates binding and trafficking of calsyntenin-1. J Cell Sci *124*, 1032-1042.

Valnegri, P., Montrasio, C., Brambilla, D., Ko, J., Passafaro, M., and Sala, C. (2011). The X-linked intellectual disability protein IL1RAPL1 regulates excitatory synapse formation by binding PTPdelta and RhoGAP2. Hum Mol Genet *20*, 4797-4809.

van Os, J., and Kapur, S. (2009). Schizophrenia. Lancet *374*, 635-645.

Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Sudhof, T.C., and Brose, N. (2006). Neuroligins determine synapse maturation and function. Neuron *51*, 741-754.

Varoqueaux, F., Jamain, S., and Brose, N. (2004). Neuroligin 2 is exclusively localized to inhibitory synapses. Eur J Cell Biol *83*, 449-456.

Varoqueaux, F., Sigler, A., Rhee, J.S., Brose, N., Enk, C., Reim, K., and Rosenmund, C. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A *99*, 9037-9042.

Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M.*, et al.* (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science *287*, 864-869.

Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P.*, et al.* (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell *65*, 905-914.

Vogt, L., Schrimpf, S.P., Meskenaite, V., Frischknecht, R., Kinter, J., Leone, D.P., Ziegler, U., and Sonderegger, P. (2001). Calsyntenin-1, a proteolytically processed postsynaptic membrane protein with a cytoplasmic calcium-binding domain. Mol Cell Neurosci *17*, 151-166.

Waites, C.L., Craig, A.M., and Garner, C.C. (2005). Mechanisms of vertebrate synaptogenesis. Annu Rev Neurosci *28*, 251-274.

Waites, C.L., and Garner, C.C. (2011). Presynaptic function in health and disease. Trends Neurosci *34*, 326-337.

Walsh, T., McClellan, J.M., McCarthy, S.E., Addington, A.M., Pierce, S.B., Cooper, G.M., Nord, A.S., Kusenda, M., Malhotra, D., Bhandari, A.*, et al.* (2008). Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. Science *320*, 539-543.

Wang, C.Y., Chang, K., Petralia, R.S., Wang, Y.X., Seabold, G.K., and Wenthold, R.J. (2006). A novel family of adhesion-like molecules that interacts with the NMDA receptor. J Neurosci *26*, 2174-2183.

Wang, X., McCoy, P.A., Rodriguiz, R.M., Pan, Y., Je, H.S., Roberts, A.C., Kim, C.J., Berrios, J., Colvin, J.S., Bousquet-Moore, D.*, et al.* (2011). Synaptic dysfunction and abnormal behaviors in mice lacking major isoforms of Shank3. Hum Mol Genet *20*, 3093-3108.

Wang, X., Su, H., and Bradley, A. (2002a). Molecular mechanisms governing Pcdh-gamma gene expression: evidence for a multiple promoter and cis-alternative splicing model. Genes & Development *16*, 1890-1905.

Wang, X., Weiner, J.A., Levi, S., Craig, A.M., Bradley, A., and Sanes, J.R. (2002b). Gamma protocadherins are required for survival of spinal interneurons. Neuron *36*, 843-854.

Wang, Z., Wang, B., Yang, L., Guo, Q., Aithmitti, N., Songyang, Z., and Zheng, H. (2009). Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis. J Neurosci *29*, 10788-10801.

Watkins, J.C., and Evans, R.H. (1981). Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol *21*, 165-204.

Weeber, E.J., Jiang, Y.H., Elgersma, Y., Varga, A.W., Carrasquillo, Y., Brown, S.E., Christian, J.M., Mirnikjoo, B., Silva, A., Beaudet, A.L.*, et al.* (2003). Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. J Neurosci *23*, 2634-2644.

White, J.M. (2003). ADAMs: modulators of cell-cell and cell-matrix interactions. Curr Opin Cell Biol *15*, 598-606.

Whittaker, V.P., and Gray, E.G. (1962). The synapse: biology and morphology. Br Med Bull *18*, 223-228.

Williams, M.E., Wilke, S.A., Daggett, A., Davis, E., Otto, S., Ravi, D., Ripley, B., Bushong, E.A., Ellisman, M.H., Klein, G.*, et al.* (2011). Cadherin-9 regulates synapse-specific differentiation in the developing hippocampus. Neuron *71*, 640-655.

Woo, J., Kwon, S.K., Choi, S., Kim, S., Lee, J.R., Dunah, A.W., Sheng, M., and Kim, E. (2009). Trans-synaptic adhesion between NGL-3 and LAR regulates the formation of excitatory synapses. Nat Neurosci *12*, 428-437.

Wright, I.C., Rabe-Hesketh, S., Woodruff, P.W., David, A.S., Murray, R.M., and Bullmore, E.T. (2000). Meta-analysis of regional brain volumes in schizophrenia. Am J Psychiatry *157*, 16-25.

Wu, Q., and Maniatis, T. (1999). A striking organization of a large family of human neural cadherin-like cell adhesion genes. Cell *97*, 779-790.

Xu, B., Woodroffe, A., Rodriguez-Murillo, L., Roos, J.L., van Rensburg, E.J., Abecasis, G.R., Gogos, J.A., and Karayiorgou, M. (2009). Elucidating the genetic architecture of familial schizophrenia using rare copy number variant and linkage scans. Proc Natl Acad Sci U S A *106*, 16746-16751.

Xu, D., Hopf, C., Reddy, R., Cho, R.W., Guo, L., Lanahan, A., Petralia, R.S., Wenthold, R.J., O'Brien, R.J., and Worley, P. (2003). Narp and NP1 form heterocomplexes that function in developmental and activity-dependent synaptic plasticity. Neuron *39*, 513-528.

Xu, J., Xiao, N., and Xia, J. (2010). Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1. Nat Neurosci *13*, 22-24.

Yagi, T., and Takeichi, M. (2000). Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. Genes & Development *14*, 1169-1180.

Yamada, S., Pokutta, S., Drees, F., Weis, W.I., and Nelson, W.J. (2005). Deconstructing the cadherin-catenin-actin complex. Cell *123*, 889-901.

Yamagata, M., Herman, J.P., and Sanes, J.R. (1995). Lamina-specific expression of adhesion molecules in developing chick optic tectum. J Neurosci *15*, 4556-4571.

Yamagata, M., Sanes, J.R., and Weiner, J.A. (2003). Synaptic adhesion molecules. Curr Opin Cell Biol *15*, 621-632.

Yang, P., Baker, K.A., and Hagg, T. (2006). The ADAMs family: coordinators of nervous system development, plasticity and repair. Prog Neurobiol *79*, 73-94.

Yashiro, K., Riday, T.T., Condon, K.H., Roberts, A.C., Bernardo, D.R., Prakash, R., Weinberg, R.J., Ehlers, M.D., and Philpot, B.D. (2009). Ube3a is required for experience-dependent maturation of the neocortex. Nat Neurosci *12*, 777-783.

Yasumura, M., Yoshida, T., Lee, S.J., Uemura, T., Joo, J.Y., and Mishina, M. (2011). Glutamate receptor delta1 induces preferentially inhibitory presynaptic differentiation of cortical neurons by interacting with neurexins through cerebellin precursor protein subtypes. J Neurochem.

Yi, J.J., and Ehlers, M.D. (2007). Emerging roles for ubiquitin and protein degradation in neuronal function. Pharmacol Rev *59*, 14-39.

Yoshida, T., Shiroshima, T., Lee, S.J., Yasumura, M., Uemura, T., Chen, X., Iwakura, Y., and Mishina, M. (2012). Interleukin-1 Receptor Accessory Protein Organizes Neuronal Synaptogenesis as a Cell Adhesion Molecule. Journal of Neuroscience *32*, 2588-2600.

Yoshida, T., Yasumura, M., Uemura, T., Lee, S.J., Ra, M., Taguchi, R., Iwakura, Y., and Mishina, M. (2011). IL-1 receptor accessory protein-like 1 associated with mental retardation and autism mediates synapse formation by trans-synaptic interaction with protein tyrosine phosphatase delta. J Neurosci *31*, 13485-13499.

Zhai, R.G., Vardinon-Friedman, H., Cases-Langhoff, C., Becker, B., Gundelfinger, E.D., Ziv, N.E., and Garner, C.C. (2001). Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. Neuron *29*, 131-143.

Zhang, C., Atasoy, D., Arac, D., Yang, X., Fucillo, M.V., Robison, A.J., Ko, J., Brunger, A.T., and Sudhof, T.C. (2010). Neurexins physically and functionally interact with GABA(A) receptors. Neuron *66*, 403-416.

Zhang, W., Rohlmann, A., Sargsyan, V., Aramuni, G., Hammer, R.E., Sudhof, T.C., and Missler, M. (2005). Extracellular domains of alpha-neurexins participate in regulating synaptic transmission by selectively affecting N- and P/Q-type Ca2+ channels. J Neurosci *25*, 4330-4342.

Zhiling, Y., Fujita, E., Tanabe, Y., Yamagata, T., Momoi, T., and Momoi, M.Y. (2008). Mutations in the gene encoding CADM1 are associated with autism spectrum disorder. Biochem Biophys Res Commun *377*, 926-929.

Zoghbi, H.Y. (2003). Postnatal neurodevelopmental disorders: meeting at the synapse? Science *302*, 826-830.